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<b>(54) Title:</b> OPIATE, CANNABINOID, AND ESTROGEN RECEPTORS		
<b>(57) Abstract</b>		
<p>The present invention relates generally to mu3 opiate receptors, cannabinoid receptors, and estrogen surface receptors (ESRs). Specifically, the invention provides methods and materials for identifying mu3 opiate receptor agonists and antagonists, cannabinoid receptor agonists and antagonists, and ESR agonists and antagonists. In addition, the invention provides an isolated nucleic acid molecule that encodes a mu3 opioid receptor, a host cell containing an isolated nucleic acid molecule that encodes a mu3 opioid receptor, and an isolated mu3 opioid receptor polypeptide. Further, the invention provides methods and materials for treating cancers, inflammatory conditions, sepsis conditions, viral infections, and cardiovascular diseases.</p>		

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## OPIATE, CANNABINOID, AND ESTROGEN RECEPTORS

### Statement as to Federally Sponsored Research

Funding for the work described herein was provided by the federal  
5 government, which may have certain rights in the invention.

## BACKGROUND

### *1. Technical Field*

The invention relates to methods and materials involved in the activation  
and inhibition of opiate, cannabinoid, and estrogen receptors. Specifically, the  
10 invention relates to mu3 opiate receptors, cannabinoid receptors, and estrogen  
surface receptors, and the biological responses induced by such receptors.

### *2. Background Information*

Three general classes of cell surface opioid receptors (kappa, delta, and  
mu) have been described based on ligand specificity. Opioid receptors exhibiting  
15 high binding specificity for morphine have been designated mu opioid receptors.  
Detailed analysis of mu opioid receptors from various tissue has revealed the  
existence of multiple mu opioid receptor subtypes. In fact, the cDNA encoding the  
mu1 opioid receptor subtype has been identified. Oligonucleotides complementary  
to some, but not all, exons of the mu1 opioid receptor can block the effects  
20 mediated by the mu1 and mu2 receptor subtypes. Thus, the mu1 and mu2 opioid  
receptor subtypes appear to share exon sequences, as would be expected of splice  
variants. Supporting the idea of alternative splicing is the finding of a single mu  
gene in mouse chromosomal DNA. In addition, a novel rat brain mu opioid  
receptor subtype, designated rMOR1B, has been identified. This receptor is  
25 identical to the rat mu1 opioid receptor at its N-terminus but differs in its length  
and sequence at the C-terminus. Further, affinity studies demonstrated that the

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substrate specificity of rMOR1B is similar to that of the rat mu1 opioid receptor, but rMOR1B is more resistant to agonist-induced desensitization and has a different expression pattern in brain. The presence of another opiate receptor, designated mu3 opiate receptor, has been demonstrated pharmacologically. This mu3 opiate  
5 receptor is opioid peptide insensitive and opiate alkaloid selective. In addition, detailed binding analysis indicates that the mu3 opiate receptor is expressed by immune tissues (e.g., human monocytes and granulocytes).

Cannabinoids induce physiological activities similar to those induced by morphine. Cannabinoids, however, induce these activities by interacting with  
10 specific cannabinoid receptors that are structurally distinct from opioid receptors. To date, two subtypes of G-protein-coupled cannabinoid receptors have been identified: CB1 and CB2. These two cannabinoid receptor subtypes are expressed by different tissues and have different pharmacological properties. For example, the CB1 receptor is expressed in brain and endothelial tissue while the CB2  
15 receptor is expressed in the immune system. In addition, SR 141716A is a CB1 receptor antagonist while SR 144528 is a CB2 receptor antagonist.

Estrogen, like morphine, appears to have multiple receptors. In fact, two different cDNA clones have been identified: one encoding estrogen receptor-alpha (ER-alpha) and the other encoding estrogen receptor-beta (ER-beta). In  
20 addition, many variants of ER-beta have been described, including human ER-beta isotypes 1 through 5. Unlike the mu opioid receptor subtypes, however, these estrogen receptors are intracellular nuclear receptors. Presumably, each of these intracellular estrogen receptors, upon interaction with estrogen, mediate biological responses by interacting directly with DNA. Tamoxifen is a lipophilic anti-  
25 estrogen compound that can inhibit the interaction of estrogen with intracellular nuclear receptors.

## SUMMARY

The present invention relates generally to mu3 opiate receptors, cannabinoid receptors, and estrogen surface receptors (ESRs). Specifically, the

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invention provides methods and materials for identifying mu3 opiate receptor agonists and antagonists, cannabinoid receptor agonists and antagonists, and ESR agonists and antagonists. In addition, the invention provides an isolated nucleic acid molecule that encodes a mu3 opioid receptor, a host cell containing an isolated  
5 nucleic acid molecule that encodes a mu3 opioid receptor, and an isolated mu3 opioid receptor polypeptide. Further, the invention provides methods and materials for treating cancers, inflammatory conditions, sepsis conditions, viral infections, and cardiovascular diseases.

The present invention is based on the discovery of a cell surface  
10 receptor for estrogen. Specifically, this cell surface receptor for estrogen, designated estrogen surface receptor 1 (ESR1), exhibits ligand specificity for estrogen and 17 $\beta$ -estradiol (E2) as well as E2 conjugated to bovine serum albumen (E2-BSA). In addition, ESR1 is tamoxifen sensitive. In other words, tamoxifen can inhibit the stimulatory effects of ESR1 agonists such as estrogen and E2.  
15 Thus, tamoxifen is an ESR1 antagonist. Further, ESR1 is expressed by human endothelial cells. Moreover, the ESR1-mediated biological responses induced by ESR1 agonists include changes in intracellular calcium concentration and nitric oxide release. The existence of a cell surface receptor for estrogen has significant medical implications. For example, plasma membrane impermeable compounds  
20 can be used to influence the biological effects of estrogen. Taken together, the discovery of ESR1 and the biological responses mediated by ESR1 provides methods and materials for modulating calcium and nitric oxide regulated mechanisms. As described herein, modulating calcium and nitric oxide regulated mechanisms can be used to treat cancers, inflammatory conditions, sepsis  
25 conditions, viral infections, and cardiovascular diseases.

The present invention is also based on the discovery of several assays for identifying mu3 opiate receptor, cannabinoid receptor, or ESR agonists and antagonists. Specifically, the assays involve monitoring at least one biological response induced by mu3 opiate receptors, cannabinoid receptors, or ESRs. Such  
30 biological responses include changes in intracellular calcium concentration and

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nitric oxide release. Thus, the assays can be configured to monitor intracellular calcium concentration, nitric oxide release, or both. The assays are particularly advantageous since the biological responses induced by mu3 opiate receptors, cannabinoid receptors, or ESRs can be detected within seconds of applying an agonist. Thus, many test molecules can be screened rapidly for the ability to either stimulate or inhibit mu3 opiate receptor, cannabinoid receptor, or ESR activities. In addition, the assays are specific for the particular receptor subtype. For example, stimulation or inhibition of mu3 opiate receptor activity can be easily distinguished from effects operating through mu1 or mu2 opioid receptors. Likewise, stimulation or inhibition of CB1 activity can be easily distinguished from effects operating through CB2, and stimulation or inhibition of ESR activity can be easily distinguished from effects operating through the intracellular nuclear receptors for estrogen (e.g., ER-alpha and ER-beta). Further, any results generated from an assay that, for example, monitors intracellular calcium concentration can be easily confirmed by performing an assay that monitors nitric oxide release. Having the ability to confirm a particular test molecule's ability to stimulate or inhibit mu3 opiate receptor, cannabinoid receptor, or ESR activity provides a powerful tool for reliably identifying receptor agonists and antagonists. Taken together, the assays described herein can be used to identify mu3 opiate receptor, cannabinoid receptor, or ESR agonists and antagonists rapidly, specifically, and reliably.

In addition, the present invention is based on the discovery of an isolated nucleic acid molecule that encodes a mu3 opiate receptor. Specifically, the mu3 opiate receptor is a cell surface receptor that exhibits specificity for morphine while being opioid peptide insensitive. In addition, the interaction of morphine with the mu3 opiate receptor induces changes in intracellular calcium concentration and nitric oxide release. Isolated nucleic acid molecules that encode the mu3 opioid receptor, isolated mu3 opioid receptor polypeptides, and host cells containing such isolated nucleic acid molecules are particularly useful to research scientists since these materials allow scientists to explore, for example, the

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interactions of morphine with the mu3 opiate receptor, the molecular mechanisms by which morphine binding induces intracellular calcium concentration changes, and the relationships of the mu3 opiate receptor with other mu opioid receptors. In addition, these materials can be used to transform mu3 opiate receptor negative  
5 cells into mu3 opiate receptor positive cells.

Further, the present invention is based on the discovery of relationships between mu3 opiate receptor, cannabinoid receptor, and ESR activities and various disease conditions. Specifically, disease conditions such as cancer can be treated with specific mu3 opiate receptor, cannabinoid receptor, or ESR antagonists, either  
10 individually or in various combinations. In addition, disease conditions such as inflammatory conditions, sepsis conditions, viral infections, and cardiovascular diseases can be treated with specific mu3 opiate receptor, cannabinoid receptor, or ESR agonists, either individually or in various combinations.

In general, the invention features a method for identifying a mu3 opiate  
15 receptor agonist. This method involves contacting a cell (e.g., cancer cell) with a test molecule and determining if the test molecule induces a mu3 opiate receptor-mediated response in the cell in a mu3 opiate receptor-specific manner. The cell expresses a mu3 opiate receptor (e.g., a human mu3 opiate receptor) and the test molecule is a molecule other than morphine or dihydromorphine. The determining  
20 step can involve monitoring nitric oxide synthase activity in the cell. For example, nitric oxide synthase activity can be monitored by detecting nitric oxide release from the cell. A nitric oxide-specific amperometric probe can be used to detect the nitric oxide release. The determining step can involve monitoring intracellular calcium levels within the cell. For example, a fluorescent ion indicator (e.g., Fura-  
25 2) can be used to monitor the intracellular calcium levels. In addition, the determining step can involve monitoring both nitric oxide synthase activity and intracellular calcium levels in the cell.

In another embodiment, the invention features a method for identifying a mu3 opiate receptor antagonist. The method involves contacting a cell (e.g., cancer  
30 cell) with a test molecule and a mu3 opiate receptor agonist (e.g., morphine or

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dihydromorphine), and determining if the test molecule influences (e.g., reduces) induction of a mu3 opiate receptor-mediated response in the cell by the mu3 opiate receptor agonist. The cell expresses a mu3 opiate receptor, and the test molecule is a molecule other than naloxone or naltrexone. The determining step can involve  
5 monitoring nitric oxide synthase activity in the cell. For example, nitric oxide synthase activity can be monitored by detecting nitric oxide release from the cell. A nitric oxide-specific amperometric probe can be used to detect the nitric oxide release. The determining step can involve monitoring intracellular calcium levels within the cell. For example, a fluorescent ion indicator (e.g., Fura-2) can be used  
10 to monitor the intracellular calcium levels. In addition, the determining step can involve monitoring both nitric oxide synthase activity and intracellular calcium levels in the cell.

Another embodiment of the invention features a method for identifying a cannabinoid receptor agonist. The method involves contacting a cell (e.g.,  
15 endothelial cell) with a test molecule, and determining if the test molecule induces a cannabinoid receptor-mediated response in the cell in a cannabinoid receptor-specific manner. The cell expresses a cannabinoid receptor (e.g., a human cannabinoid receptor such as CB1), and the test molecule is a molecule other than anandamide. The determining step can involve monitoring nitric oxide synthase  
20 activity in the cell. For example, nitric oxide synthase activity can be monitored by detecting nitric oxide release from the cell. A nitric oxide-specific amperometric probe can be used to detect the nitric oxide release. The determining step can involve monitoring intracellular calcium levels within the cell. For example, a fluorescent ion indicator (e.g., Fura-2) can be used to monitor the  
25 intracellular calcium levels. In addition, the determining step can involve monitoring both nitric oxide synthase activity and intracellular calcium levels in the cell.

Another embodiment of the invention features a method for identifying a cannabinoid receptor antagonist. The method involves contacting a cell (e.g.,  
30 endothelial cell) with a test molecule and a cannabinoid receptor agonist (e.g.,



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anandamide), and determining if the test molecule influences (e.g., reduces) induction of a cannabinoid receptor-mediated response in the cell by said cannabinoid receptor agonist. The cell expresses a cannabinoid receptor (e.g., CB1 receptor), and the test molecule is a molecule other than SR 141716A. The

5 determining step can involve monitoring nitric oxide synthase activity in the cell. For example, nitric oxide synthase activity can be monitored by detecting nitric oxide release from the cell. A nitric oxide-specific amperometric probe can be used to detect the nitric oxide release. The determining step can involve monitoring intracellular calcium levels within the cell. For example, a fluorescent

10 ion indicator (e.g., Fura-2) can be used to monitor the intracellular calcium levels. In addition, the determining step can involve monitoring both nitric oxide synthase activity and intracellular calcium levels in the cell.

Another embodiment of the invention features a method for identifying an estrogen surface receptor agonist. The method involves contacting a cell (e.g.,

15 endothelial cell) with a test molecule, and determining if the test molecule induces an estrogen surface receptor-mediated response in the cell in an estrogen surface receptor-specific manner. The cell expresses an estrogen surface receptor such as a human estrogen surface receptor. The estrogen surface receptor can be ESR1. The test molecule can be plasma membrane impermeable. The determining step can

20 involve monitoring nitric oxide synthase activity in the cell. For example, nitric oxide synthase activity can be monitored by detecting nitric oxide release from the cell. A nitric oxide-specific amperometric probe can be used to detect the nitric oxide release. The determining step can involve monitoring intracellular calcium levels within the cell. For example, a fluorescent ion indicator (e.g., Fura-2) can

25 be used to monitor the intracellular calcium levels. In addition, the determining step can involve monitoring both nitric oxide synthase activity and intracellular calcium levels in the cell.

Another embodiment of the invention features a method for identifying an estrogen surface receptor antagonist. The method involves contacting a cell

30 with a test molecule and an estrogen surface receptor agonist, and determining if

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the test molecule influences (e.g., reduces) induction of an estrogen surface receptor-mediated response in the cell by the estrogen surface receptor agonist. The cell expresses an estrogen surface receptor such as a human estrogen surface receptor. The estrogen surface receptor can be ESR1. The test molecule can be plasma membrane impermeable. The estrogen surface receptor agonist can be estrogen,  $17\beta$ -estradiol, or  $17\beta$ -estradiol-BSA. The determining step can involve monitoring nitric oxide synthase activity in the cell. For example, nitric oxide synthase activity can be monitored by detecting nitric oxide release from the cell. A nitric oxide-specific amperometric probe can be used to detect the nitric oxide release. The determining step can involve monitoring intracellular calcium levels within the cell. For example, a fluorescent ion indicator (e.g., Fura-2) can be used to monitor the intracellular calcium levels. In addition, the determining step can involve monitoring both nitric oxide synthase activity and intracellular calcium levels in the cell.

In another aspect, the invention features an isolated nucleic acid molecule having first and second nucleic acid sequences. The first nucleic acid sequence is substantially homologous to SEQ ID NO:1, and the second nucleic acid sequence is substantially homologous to SEQ ID NO:2. The first and second nucleic acid sequences are separated by more than about 1500 nucleotides. The isolated nucleic acid molecule can encode a  $\mu 3$  opiate receptor polypeptide. In addition, the isolated nucleic acid molecule can have a third nucleic acid sequence at least about 80 percent identical to SEQ ID NO:5. The third nucleic acid sequence is located between the first and second nucleic acid sequences.

Another aspect of the invention features a host cell containing an isolated nucleic acid molecule. The isolated nucleic acid molecule has a first and second nucleic acid sequence. The first nucleic acid sequence is substantially homologous to SEQ ID NO:1, and the second nucleic acid sequence is substantially homologous to SEQ ID NO:2. The first and second nucleic acid sequences are separated by more than about 1500 nucleotides.

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Another aspect of the invention features an isolated polypeptide having an amino acid sequence at least about 80 percent identical to SEQ ID NO:6. The polypeptide is between 403 and 600 amino acid residues.

Another aspect of the invention features a method for treating a mammal  
5 (e.g., human) having cancer. The method involves administering a mu3 opiate receptor antagonist to the mammal such that a mu3 opiate receptor-mediated response is reduced. The reduction of the mu3 opiate receptor-mediated response promotes anti-tumor activity in the mammal. The cancer can be lung cancer, breast cancer, prostate cancer, colon cancer, carcinoma, leukemia, or melanoma. The mu3  
10 opiate receptor-mediated response can be a change in intracellular calcium concentration in a cell (e.g., cancer cell) within the mammal. The mu3 opiate receptor-mediated response can be a change in the amount of nitric oxide released from a cell within the mammal. The method can involve administering a cannabinoid receptor antagonist (e.g., a CB1 receptor antagonist) to the mammal  
15 such that a cannabinoid receptor-mediated response is reduced. The method can involve administering an estrogen surface receptor antagonist (e.g., an ESR1 antagonist) to the mammal such that an estrogen surface receptor-mediated response is reduced. The estrogen surface receptor antagonist can be plasma membrane impermeable, for example, tamoxifen coupled to bovine serum albumin.

20 In another embodiment, the invention features a method for treating a mammal having cancer. The method involves administering a cannabinoid receptor antagonist (e.g., a CB1 receptor antagonist) to the mammal such that a cannabinoid receptor-mediated response is reduced. The reduction of the cannabinoid receptor-mediated response promotes anti-tumor activity in the mammal.

25 Another embodiment of the invention features a method for treating a mammal having cancer. The method involves administering an estrogen surface receptor antagonist (e.g., an ESR1 antagonist) to the mammal such that an estrogen surface receptor-mediated response is reduced. The estrogen surface receptor antagonist is plasma membrane impermeable. In addition, the reduction of the

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estrogen surface receptor-mediated response promotes anti-tumor activity in the mammal.

Another embodiment of the invention features a method for treating a mammal having an inflammatory condition. The method involves administering a mu3 opiate receptor agonist to the mammal such that a mu3 opiate receptor-mediated response is induced. In addition, the induction of the mu3 opiate receptor-mediated response promotes anti-inflammatory or immunosuppressive activity in the mammal. The inflammatory condition can be arthritis, pericarditis, vasculitis, lupus, bronchitis, or phrenitis. The method can involve administering a cannabinoid receptor agonist to the mammal such that a cannabinoid receptor-mediated response is induced. The method can involve administering an estrogen surface receptor agonist to the mammal such that an estrogen surface receptor-mediated response is induced.

Another embodiment of the invention features a method for treating a mammal having an inflammatory condition. The method involves administering a cannabinoid receptor agonist to the mammal such that a cannabinoid receptor-mediated response is induced. The induction of the cannabinoid receptor-mediated response promotes anti-inflammatory or immunosuppressive activity in the mammal.

Another embodiment of the invention features a method for treating a mammal having an inflammatory condition. The method involves administering an estrogen surface receptor agonist to the mammal such that an estrogen surface receptor-mediated response is induced. The induction of the estrogen surface receptor-mediated response promotes anti-inflammatory or immunosuppressive activity in the mammal.

Another embodiment of the invention features a method for treating a mammal having sepsis. The method involves administering a mu3 opiate receptor agonist to the mammal such that a mu3 opiate receptor-mediated response is induced. The induction of the mu3 opiate receptor-mediated response reduces septic shock in the mammal. The method can involve administering a cannabinoid

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receptor agonist to the mammal such that a cannabinoid receptor-mediated response is induced. The method can involve administering an estrogen surface receptor agonist to the mammal such that an estrogen surface receptor-mediated response is induced.

5 Another embodiment of the invention features a method for treating a mammal having sepsis. The method involves administering a cannabinoid receptor agonist to the mammal such that a cannabinoid receptor-mediated response is induced. The induction of the cannabinoid receptor-mediated response reduces septic shock in the mammal.

10 Another embodiment of the invention features a method for treating a mammal having sepsis. The method involves administering an estrogen surface receptor agonist to the mammal such that an estrogen surface receptor-mediated response is induced. The induction of the estrogen surface receptor-mediated response reduces septic shock in the mammal.

15 Another embodiment of the invention features a method for treating a mammal having a viral infection (e.g., HIV infection). The method involves administering a mu3 opiate receptor agonist to the mammal such that a mu3 opiate receptor-mediated response is induced. The induction of the mu3 opiate receptor-mediated response promotes an anti-viral response in the mammal. The method  
20 can involve administering a cannabinoid receptor agonist to the mammal such that a cannabinoid receptor-mediated response is induced. The method can involve administering an estrogen surface receptor agonist to the mammal such that an estrogen surface receptor-mediated response is induced.

Another embodiment of the invention features a method for treating a  
25 mammal having a viral infection. The method involves administering a cannabinoid receptor agonist to the mammal such that a cannabinoid receptor-mediated response is induced. The induction of the cannabinoid receptor-mediated response promotes an anti-viral response in the mammal.

Another embodiment of the invention features a method for treating a  
30 mammal having a viral infection. The method involves administering an estrogen

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surface receptor agonist to the mammal such that an estrogen surface receptor-mediated response is induced. The induction of the estrogen surface receptor-mediated response promotes an anti-viral response in the mammal.

Another embodiment of the invention features a method for treating a  
5 mammal having cardiovascular disease. The method involves administering an estrogen surface receptor agonist to the mammal such that an estrogen surface receptor-mediated response is induced. The estrogen surface receptor agonist is plasma membrane impermeable. The induction of the estrogen surface receptor-mediated response can reduce or prevent atherosclerosis in the mammal.

10 Another embodiment of the invention features a method for treating a mammal with a  $\mu_3$  opiate receptor agonist such that  $\mu_3$  opiate receptor-mediated nitric oxide release is suppressed. The method involves administering an opioid receptor agonist to the mammal followed by administering the  $\mu_3$  opiate receptor agonist. The opioid receptor agonist is not a  $\mu_3$  opiate receptor agonist. The  
15 opioid receptor agonist can be DAMA,  $\beta$ -endorphin, or DAMGO. The  $\mu_3$  opiate receptor agonist can be morphine or dihydromorphine.

Another aspect of the invention features a pharmaceutical formulation containing a  $\mu_3$  opiate receptor antagonist and an estrogen surface receptor antagonist.

20 In another embodiment, the invention features a pharmaceutical formulation containing a  $\mu_3$  opiate receptor antagonist and a cannabinoid receptor antagonist.

Another embodiment of the invention features a pharmaceutical formulation containing an estrogen surface receptor antagonist and a cannabinoid  
25 receptor antagonist.

Another embodiment of the invention features a pharmaceutical formulation containing an estrogen surface receptor antagonist that is membrane impermeable.

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Another embodiment of the invention features a pharmaceutical formulation containing a mu3 opiate receptor agonist and an estrogen surface receptor agonist.

Another embodiment of the invention features a pharmaceutical  
5 formulation containing a cannabinoid receptor agonist and an estrogen surface receptor agonist.

Another aspect of the invention features the use of a mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor antagonist in the manufacture of a medicament for the treatment of cancer. The estrogen surface  
10 receptor antagonist is plasma membrane impermeable.

In another embodiment, the invention features the use of a mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor agonist in the manufacture of a medicament for the treatment of an inflammatory condition.

Another embodiment of the invention features the use of a mu3 opiate  
15 receptor, cannabinoid receptor, or estrogen surface receptor agonist in the manufacture of a medicament for the treatment of sepsis.

Another embodiment of the invention features the use of a mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor agonist in the manufacture of a medicament for the treatment of a viral infection.

Another embodiment of the invention features the use of an estrogen  
20 surface receptor agonist in the manufacture of a medicament for the treatment of cardiovascular disease. The estrogen surface receptor agonist is plasma membrane impermeable.

Another aspect of the invention features an article of manufacture  
25 containing packaging material and a mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor antagonist contained within the packaging material. The packaging material contains a label or package insert indicating that the mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor antagonist can be administered to a mammal to treat cancer. The estrogen surface receptor antagonist  
30 is plasma membrane impermeable.

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In another embodiment, the invention features an article of manufacture containing packaging material and a mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor agonist contained within the packaging material. The packaging material contains a label or package insert indicating that the mu3 opiate  
5 receptor, cannabinoid receptor, or estrogen surface receptor agonist can be administered to a mammal to treat an inflammatory condition.

Another embodiment of the invention features an article of manufacture containing packaging material and a mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor agonist contained within the packaging material. The  
10 packaging material contains a label or package insert indicating that the mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor agonist can be administered to a mammal to reduce septic shock.

Another embodiment of the invention features an article of manufacture containing packaging material and a mu3 opiate receptor, cannabinoid receptor, or  
15 estrogen surface receptor agonist contained within the packaging material. The packaging material contains a label or package insert indicating that the mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor agonist can be administered to a mammal to treat a viral infection.

Another embodiment of the invention features an article of manufacture  
20 containing packaging material and an estrogen surface receptor agonist contained within the packaging material. The packaging material contains a label or package insert indicating that the estrogen surface receptor agonist can be administered to a mammal to treat cardiovascular disease. The estrogen surface receptor agonist is plasma membrane impermeable.

25 Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All  
30 publications, patent applications, patents, and other references mentioned herein are



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incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

### DESCRIPTION OF DRAWINGS

Figure 1 is a graph plotting NO release verses time for granulocytes, normal lung tissue, and non-small cell lung carcinoma tissue exposed to the indicated compounds.

Figure 2 is a graph plotting NO release verses time for vascular tissue exposed to opioid peptides followed by morphine treatment (100 nM).

Figure 3 is a graph plotting real-time intracellular calcium concentration verses time for human arterial endothelial cells treated with morphine (1  $\mu$ M), DAMGO (1  $\mu$ M), or naloxone (1  $\mu$ M) plus morphine (1  $\mu$ M).

Figure 4 is a graph plotting real-time intracellular calcium concentration and NO release verses time for human arterial endothelial cells treated with morphine (1  $\mu$ M) or anandamide (1  $\mu$ M).

Figure 5 is a graph plotting real-time intracellular calcium concentration verses time for human arterial endothelial cells treated with anandamide (1  $\mu$ M) or SR 141716A (1  $\mu$ M) plus anandamide (1  $\mu$ M).

Figure 6 is a graph plotting real-time nitric oxide release and intracellular calcium concentration verses time for cultured human endothelial cells treated with 17 $\beta$ -estradiol (E; 1 nM), tamoxifen (T, 1 nM) plus 17 $\beta$ -estradiol (1 nM), or ICI 182,780 (ICI, 1 nM) plus 17 $\beta$ -estradiol (1 nM). The curve corresponding to the cells treated first with tamoxifen and then 17 $\beta$ -estradiol was a straight line raised for better visualization.

Figure 7 is a graph plotting NO release verses drug concentration for ITA fragments (A) and cultured arterial endothelial cells (B) exposed to 17 $\beta$ -estradiol, E<sub>2</sub>-BSA, or vehicle only.

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Figure 8 is a graph plotting nitric oxide release verses time for cultured arterial endothelial cells treated with  $17\beta$ -estradiol (17 B-E; 1 nM), tamoxifen (1 nM), E2-BSA (1 nM),  $17\beta$ -estradiol (17 B-E; 1 nM) plus tamoxifen (Tam, 1 nM), or E2-BSA (1 nM) plus tamoxifen (1 nM).

5           Figure 9 is a graph plotting nitric oxide release verses time for cultured arterial endothelial cells treated with ICI 182,780 (ICI; 1 nM), E2-BSA (1 nM), or E2-BSA (1 nM) plus ICI 182,780 (1 nM).

Figure 10 is a graph plotting real-time intracellular calcium concentration and NO release verses time for cultured human arterial endothelial  
10 cells treated with E<sub>2</sub>-BSA (1 nM).

### DETAILED DESCRIPTION

The invention provides methods and materials for identifying mu3 opiate receptor, cannabinoid receptor, and ESR agonists and antagonists. In addition, the invention provides an isolated nucleic acid molecule that encodes a mu3 opioid  
15 receptor, a host cell containing an isolated nucleic acid molecule that encodes a mu3 opioid receptor, and an isolated mu3 opioid receptor polypeptide. Further, the invention provides methods and materials for treating cancers, inflammatory conditions, sepsis conditions, viral infections, and cardiovascular diseases.

#### *Identifying receptor agonists*

20           A receptor agonist is any molecule that specifically interacts with a receptor and initiates a biological response mediated by that receptor. For example, an agonist for receptor X can be any molecule that induces an X receptor-mediated response in an X receptor-specific manner. Thus, a mu3 opiate receptor agonist is any molecule that specifically interacts with a mu3 opiate receptor and initiates a  
25 mu3 opiate receptor-mediated response. Mu3 opiate receptor-mediated responses include, without limitation, changes in intracellular calcium concentration and nitric oxide release. In addition, a cannabinoid receptor agonist is any molecule that specifically interacts with a cannabinoid receptor and initiates a cannabinoid

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receptor-mediated response. Cannabinoid receptor-mediated responses include, without limitation, changes in intracellular calcium concentration and nitric oxide release. Further, an ESR agonist is any molecule that specifically interacts with an ESR and initiates an ESR-mediated response. ESR-mediated responses include, without limitation, changes in intracellular calcium concentration and nitric oxide release.

The specificity of the interactions of receptor agonists with mu3 opiate receptors, cannabinoid receptors, and ESRs can be determined using known mu3 opiate receptor, cannabinoid receptor, and ESR antagonists, respectively. For example, a test molecule that induces a biological response that a mu3 opiate receptor mediates can be identified as a mu3 opiate receptor agonist if a mu3 opiate receptor antagonist inhibits the induction of that particular biological response. In addition, the specificity of agonist-receptor interactions can be demonstrated using heterologous expression systems, receptor binding analyses, or any other method that provides a measure of agonist-receptor interaction.

Agonists for a particular receptor can be identified by contacting cells that express that particular receptor with a test molecule, and determining if that test molecule induces a response mediated by that particular receptor in a manner specific for that receptor. For example, a mu3 opiate receptor agonist can be identified by contacting mu3 opiate receptor positive cells with a test molecule, and determining if that test molecule induces a mu3 opiate receptor response in those cells in a mu3 opiate receptor-specific manner. A test molecule can be any molecule having any chemical structure. For example, a test molecule can be a polypeptide, carbohydrate, lipid, amino acid, nucleic acid, fatty acid, steroid. In addition, a test molecule can be lipophilic, hydrophilic, plasma membrane permeable, or plasma membrane impermeable.

The invention provides several assays that can be used to identify receptor agonists. Such assays involve monitoring at least one of the biological responses mediated by a mu3 opiate receptor, cannabinoid receptor, or ESR. As described herein, mu3 opiate receptor-, cannabinoid receptor-, and ESR-mediated

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responses include, without limitation, increases in intracellular calcium concentration and nitric oxide release. Thus, a  $\mu_3$  opiate receptor, cannabinoid receptor, or ESR agonist can be identified using an assay that monitors intracellular calcium concentration, nitric oxide release, or both.

- 5 Intracellular calcium concentrations can be monitored using any method. For example, intracellular calcium concentrations can be monitored using a dye that detects calcium ions. In this case, cells can be loaded with fura-2, a fluorescent dye, and monitored by dual emission microfluorimetry. The fura-2 loading process can involve washing the cells (e.g., one to four times) with incubation medium
- 10 lacking calcium. This medium can be balanced with sucrose to maintain osmolarity. After washing, the cells can be incubated (e.g., 30 minutes) with loading solution. This loading solution can contain, for example, 5  $\mu$ M fura-2/AM and a non-ionic/non-denaturing detergent such as Pluronic F-127. The non-ionic/non-denaturing detergent can help disperse the acetoxymethyl (AM) esters of
- 15 fura-2. After incubation with the loading solution, the cells can be washed (e.g., one to four times) with, for example, PBS without calcium or magnesium to remove extracellular dye.

Once loaded, the intracellular calcium concentration ( $[Ca^{2+}]_i$ ) can be calculated from the fluorescence ratio (340 and 380 nm excitation and 510 nm

20 emission wavelength) according to the following equation:  $[Ca^{2+}]_i = (R - R_{min}) k_d \beta / (R_{max} - R)$ ; where  $R$  = fluorescence ratio recorded from the cell;  $R_{min}$  = fluorescence ratio of fura-2 free acid recorded in absence of  $Ca^{2+}$ ;  $R_{max}$  = fluorescence ratio of fura-2 free acid recorded in saturating concentration of  $Ca^{2+}$ ;  $k_d$  = calcium dissociation constant of the dye; and  $\beta$  = the ratio of fluorescence of

25 fura-2 free acid in the  $Ca^{2+}$  free form to the  $Ca^{2+}$  saturated form recorded at the wavelength used in the denominator of the ratio. Using an image processing system such as a COMPIX C-640 SIMCA (Compix Inc., Mars, PA) system with an inverted microscope, images can be acquired for analysis every 0.4 seconds.

- Nitric oxide (NO) release can be monitored directly or indirectly using
- 30 any method. For example, a NO-specific amperometric probe can be used to

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measure directly the NO released from cultured cells or tissue fragments as described elsewhere (Stefano GB *et al.*, *J. Biol. Chem.* 270:30290 (1995) and Magazine HL *et al.*, *J. Immunol.* 156:4845 (1996)). Using this NO-specific probe, the concentration of NO gas in solution can be measured in real-time with, for example, a DUO 18 computer data acquisition system obtained from World Precision Instruments. Briefly, the cells or tissue fragments can be placed in a superfusion chamber containing, for example, 2 mL PBS. In addition, a micromanipulator (e.g., a micromanipulator obtained from Zeiss-Eppendorff) can be attached to the stage of a inverted microscope to aid in positioning the amperometric probe 15  $\mu\text{m}$  above the surface of a cell or tissue fragment. Prior to obtaining measurements, the amperometric probe can be calibrated by generating a standard curve using different concentrations of a nitrosothiol donor such as S-nitroso-N-acetyl-DL-penicillamine (SNAP) obtained from Sigma (St. Louis, MO). In addition, the amperometric probe can be equilibrated in the same solution (e.g., PBS) used to incubate the cells or tissue fragments during analysis.

#### *Identifying receptor antagonists*

A receptor antagonist is any molecule that specifically interacts with a receptor and inhibits a receptor agonist from initiating a biological response mediated by that receptor. For example, an antagonist for receptor X can be any molecule that inhibits an X receptor agonist from inducing an X receptor-mediated response in an X receptor-specific manner. Thus, a  $\mu_3$  opiate receptor antagonist is any molecule that specifically interacts with a  $\mu_3$  opiate receptor and inhibits a  $\mu_3$  receptor agonist from initiating a  $\mu_3$  opiate receptor-mediated response.

An antagonist for a particular receptor can be identified by contacting cells that express that particular receptor with an agonist for that receptor and a test molecule, and determining if that test molecule inhibits the receptor agonist from inducing a response mediated by that particular receptor in a manner specific for that receptor. For example, a  $\mu_3$  opiate receptor antagonist can be identified by contacting  $\mu_3$  opiate receptor positive cells with a  $\mu_3$  opiate receptor agonist

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such as morphine and a test molecule, and determining if that test molecule inhibits the mu3 opiate receptor agonist from inducing a mu3 opiate receptor response in those cells in a mu3 opiate receptor-specific manner. Again, a test molecule can be any molecule having any chemical structure. For example, a test molecule can be a polypeptide, carbohydrate, lipid, amino acid, nucleic acid, fatty acid, steroid. In addition, a test molecule can be lipophilic, hydrophilic, plasma membrane permeable, or plasma membrane impermeable.

It is to be understood that each of the assays for identifying receptor agonists described herein can be easily adapted such that receptor antagonists can be identified.

#### *Isolated nucleic acid molecules*

The term "nucleic acid" as used herein encompasses both RNA and DNA, including cDNA, genomic DNA, and synthetic (e.g., chemically synthesized) DNA. The nucleic acid can be double-stranded or single-stranded. Where single-stranded, the nucleic acid can be the sense strand or the antisense strand.

The term "isolated" as used herein with reference to nucleic acid refers to a naturally-occurring nucleic acid sequence that is not immediately contiguous with both of the sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism from which it is derived. For example, an isolated nucleic acid molecule can be, without limitation, a recombinant DNA molecule of any length, provided one of the nucleic acid sequences normally found immediately flanking that recombinant DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid molecule includes, without limitation, a recombinant DNA that exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid

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can include a recombinant DNA molecule that is part of a hybrid or fusion nucleic acid sequence.

The term "isolated" also includes any non-naturally-occurring nucleic acid sequence since non-naturally-occurring nucleic acid sequences are not found in nature and do not have immediately contiguous sequences in a naturally-occurring genome. For example, non-naturally-occurring nucleic acid sequences such as engineered nucleic acid sequences are isolated nucleic acid sequences. Engineered nucleic acid sequences can be made using common molecular cloning or chemical nucleic acid synthesis techniques. Isolated non-naturally-occurring nucleic acid sequences can be independent of other sequences or incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or the genomic DNA of a prokaryote or eukaryote. In addition, a non-naturally-occurring nucleic acid sequence can include a nucleic acid molecule that is part of a hybrid or fusion nucleic acid sequence.

An isolated nucleic acid molecule within the scope of the invention is any isolated nucleic acid molecule having a first nucleic acid sequence that is substantially homologous to 5'-GCAGAGGAGAATGTCAGATG-3' (SEQ ID NO:1) and a second nucleic acid sequence that is substantially homologous to 5'-CTAAGCTTGGTGAAGGTCGG-3' (SEQ ID NO:2) separated by more than about 1500 nucleotides (e.g., about 1550, 1600, 1650, 1700, 1750, 1800, 1850, 1900, 1950, 2000, 2050, 2100, or 2150 nucleotides). In other words, more than 1500 nucleotides are located between the first and second nucleic acid sequences. Such isolated nucleic acid molecules can encode polypeptides having the biological characteristics of a mu3 opiate receptor polypeptide as described herein. In addition, the nucleic acid sequence between the first and second nucleic acid sequences can contain a third nucleic acid sequence that is substantially homologous to 5'-GGTACTGGGAAAACCTCGTGAAGATCTGTG-3' (SEQ ID NO:3) and a fourth nucleic acid sequence that is substantially homologous to 5'-GGTCTCTAGTGTCTGACGAATTCGAGTGG-3' (SEQ ID NO:4). The third and fourth nucleic acid sequences can be separated by about 400 to about 450

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nucleotides (e.g., about 440 nucleotides). Further, the isolated nucleic acid molecules within the scope of the invention can have an internal nucleic acid sequence that is located between the first and second nucleic acid sequences and that is at least about 80 percent identical to 5'-GGTACTGGGAAAACCTGCTGAA

5 GATCTGTGTTTTTCATCTTCGCCTTCATTATGCCAGTGCTCATCATTACCGTG  
TGCTATGGACTGATGATCTTGGCCTCAAGAGTGTCGCATGCTCTCTGGC  
TCCAAAGAAAAGGACAGGAATCTTCGAAGGATCACCAGGATGGTGCTGGT  
GGTGGTGGCTGTGTTTCATCGTCTGCTGGACTCCCATTACATTTACGTCAT  
CATTAAAGCCTTGGTTACAATCCCAGAACTACGTTCCAGACTGTTTCTTG

10 GCACTTCTGCATTGCTCTAGGTTACACAAACAGCTGCCTCAACCCAGTCCT  
TTATGCATTTCTGGATGAAAACCTTCAAACGATGCTTCAGAGAGTTCTGTAT  
CCCAACCTCTTCCAACATTGAGCAACAAACTCCACTCGAATTCGTCAGA  
ACACTAGAGACC-3' (SEQ ID NO:5).

Sequences substantially homologous to a particular sequence (e.g., to

15 SEQ ID NOs:1 and 2) include, without limitation, sequences that hybridize to that particular sequence under high to moderate stringency. High stringency conditions are used to identify nucleic acids that have a high degree of homology or sequence identity to one another. High stringency conditions can include the use of a denaturing agent such as formamide during hybridization, e.g., 50% formamide

20 with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, and 75 mM sodium citrate at 42°C. Another example is the use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml),

25 0.1% sodium lauryl sulfate (SDS), and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS. Alternatively, low ionic strength and high temperature can be employed for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate (0.1X SSC); 0.1% SDS at 65°C.

Moderate stringency conditions are hybridization conditions used to

30 identify nucleic acids that have less homology or identity to one another than do



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nucleic acids identified under high stringency conditions. Moderate stringency conditions can include the use of higher ionic strength and/or lower temperatures for washing of the hybridization membrane, compared to the ionic strength and temperatures used for high stringency hybridization. For example, a wash solution  
5 comprising 0.060 M NaCl/0.0060 M sodium citrate (4X SSC) and 0.1% SDS can be used at 50°C, with a last wash in 1X SSC, at 65°C. Alternatively, a hybridization wash in 1X SSC at 37°C can be used.

The isolated nucleic acid molecules described herein can be isolated using any molecular biology technique such as those described by Sambrook J *et al.* (Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor  
10 Laboratory Press (1989)). For example, nucleic acid hybridization or PCR can be used to obtain the isolated nucleic acid molecules described herein.

#### *Host cells*

A host cell within the scope of the invention is any cell containing at  
15 least one isolated nucleic acid molecule described herein. Such cells can be prokaryotic and eukaryotic cells. It is noted that cells containing an isolated nucleic acid molecule within the scope of the invention are not required to express them. In addition, the isolated nucleic acid molecule can be integrated into the genome of the cell or maintained in an episomal state. Thus, cells can be stably or  
20 transiently transfected with a construct containing an isolated nucleic acid molecule of the invention.

Methods of introducing an isolated nucleic acid molecule into a cell, whether *in vivo* or *in vitro*, are well known to those skilled in the art. For example, calcium phosphate precipitation, electroporation, heat shock, lipofection,  
25 microinjection, and viral-mediated nucleic acid transfer are common methods of introducing nucleic acid into cells. In addition, naked DNA can be delivered directly to cells *in vivo* as describe elsewhere (U.S. Patent Number 5,580,859 and U.S. Patent Number 5,589,466 including continuations thereof). Further, nucleic acid can be introduced into cells by generating transgenic animals.

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Transgenic animals can be aquatic animals (such as fish, sharks, dolphin, and the like), farm animals (such as pigs, goats, sheep, cows, horses, rabbits, and the like), rodents (such as rats, guinea pigs, and mice), non-human primates (such as baboon, monkeys, and chimpanzees), and domestic animals (such as dogs and cats). Several techniques known in the art can be used to introduce nucleic acid into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (U.S. Patent No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten *et al.*, *Proc. Natl. Acad. Sci., USA*, 82:6148 (1985)); gene transfection into embryonic stem cells (Gossler A *et al.*, *Proc Natl Acad Sci USA* 83:9065-9069 (1986)); gene targeting into embryonic stem cells (Thompson *et al.*, *Cell*, 56:313 (1989)); nuclear transfer of somatic nuclei (Schnieke AE *et al.*, *Science* 278:2130-2133 (1997)); and electroporation of embryos (Lo CW, *Mol. Cell. Biol.*, 3:1803-1814 (1983)).

Methods of identifying cells that contain an isolated nucleic acid molecule of the invention are also well known to those skilled in the art. Such methods include, without limitation, PCR and nucleic acid hybridization techniques such as Northern and Southern analysis. In some cases, immunohistochemistry and biochemical techniques can be used to determine if a cell contains a particular isolated nucleic acid molecule by detecting the expression of a polypeptide encoded by that particular molecule.

#### *Isolated polypeptide*

An isolated polypeptide within the scope of the invention is any substantially pure polypeptide having more than about 403 amino acid residues (e.g., about 410, 420, 450, 500, 550, 600, 700, 800, or 900) and an amino acid sequence at least about 80 percent identical to: YWENLLKICVFIFAFIMPVLII TVCYGLMILRLKSVRMLSGSKEKDRNLRRITRMVLVVVAVFIVCWTPIHIVVI IKALVTIPETTFQTVSWHFCIALGYTNSCLNPVLYAFLDENFKRCFREFCIPTSS NIEQQNSTRIRQNTRD (SEQ ID NO:6). For example, such a polypeptide can be between about 403 and 600 amino acid residues. Substantially pure means the

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preparation contains at least about 60 percent (e.g., about 70, 75, 80, 85, 90, 95, or 99 percent) by weight (dry weight) of the compound of interest (e.g., a  $\mu_3$  opiate receptor).

*Treating various disease conditions*

5 Mammals having cancer can be treated with specific  $\mu_3$  opiate receptor antagonists, specific cannabinoid receptor antagonists, or specific ESR antagonists, either individually or in various combinations, to inhibit the release of nitric oxide. Inhibiting nitric oxide release can promote anti-tumor activity in mammals having cancers such as lung cancer, breast cancer, prostate cancer, colon cancer,  
10 carcinoma, leukemia, or melanoma. Such antagonists can be administered to a mammal at a concentration the specifically inhibits receptor activity. Particular concentrations for individual antagonists can be determined using standard medical and biochemical techniques.

In addition, mammals having disease conditions such as inflammatory  
15 conditions (e.g., arthritis, pericarditis, vasculitis, lupus, bronchitis, or phrenitis), sepsis conditions, viral infections (e.g., HIV infections), and cardiovascular diseases can be treated with specific  $\mu_3$  opiate receptor agonists, specific cannabinoid receptor agonists, or specific ESR agonists, either individually or in various combinations, to increase the release of nitric oxide. Increasing the release of nitric  
20 oxide can promote responses that are beneficial to the mammal. For example, nitric oxide release can promote anti-inflammatory and immunosuppressive responses, prevent septic shock, promote anti-viral activity, and reduce or prevent atherosclerosis. Such agonists can be administered to a mammal at a concentration that specifically stimulates receptor activity. Particular concentrations for  
25 individual agonists can be determined using standard medical and biochemical techniques.

Receptor agonists or antagonists can be administered by any route including, without limitation, intravenous, intraperitoneal, intramuscular, and oral administrations.

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The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

## EXAMPLES

### Example 1 - Methods and materials for mu3 opiate receptor studies

#### 5 1. Isolation of human granulocytes and tissues

Blood was obtained from the Long Island Blood Services (Melville, NY). Granulocytes were isolated from blood by standard Ficoll-Hypaque separation and the cells resuspended in RPMI medium. Microscopic examination showed a high degree of purity (e.g, about 95 to 97 percent). Tissue specimens,  
10 freshly excised lung cancer and thyroid goiter, were obtained from therapy-free patients undergoing surgical treatment. Tissue and cell samples were stored at -70°C in a guanidine-isothiocyanate solution (4M guanidine isothiocyanate, with 25mM sodium citrate, 0.5% sarcosyl, and 0.1M  $\beta$ -mercaptoethanol) until RNA extraction.

#### 15 2. RNA extraction

Total RNA was isolated using a guanidinium thiocyanate-phenol-chloroform method. The integrity of the isolated RNA was confirmed by agarose gel electrophoresis. The RNA samples were treated with DNase I, (RNase free; Boehringer Mannheim) for 30 minutes at 37°C, followed by phenol extraction.  
20 The RNA was then precipitated with ethanol and resuspended in water.

#### 3. Oligonucleotide primers

PCR was performed as a two step reaction with nested primers. The outer primer pair were designed to amplify a 1435 base pair segment of the mu1 receptor (map position 17 to 1452 based on the published 5' and 3' untranslated  
25 region; Bare LA et al., *FEBS Lett.* 354:213 (1994)). These outer primers were 5'-GCAGAGGAGAATGTCAGATG-3' (SEQ ID NO:1) and 5'-CTAAGCTTGGTGAAGGTCGG-3' (SEQ ID NO:2). The inner primer pair were

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designed to amplify a 440 base pair segment of mul (map position 896 to 1336).

This segment encodes the third extracellular loop of the mul receptor, a region important for mul receptor agonist selectivity. The inner primers were 5'-GGTACTGGGAAAACCTCGTGAAGATCTGTG-3' (SEQ ID NO:3) and 5'-

- 5 GGTCTCTAGTGTCTGACGAATTCGAGTGG-3' (SEQ ID NO:4). Each oligonucleotide primer was synthesized in an Applied Biosystems 394 DNA/RNA Synthesizer.

#### 4. Reverse-Transcriptase and polymerase chain reaction

- Complementary DNA (cDNA) was synthesized from 10 µg of total  
10 RNA using an oligo dT primer and 20 U of Avian Myeloblastosis Virus reverse transcriptase (First strand cDNA synthesis kit for RT-PCR, Boehringer Mannheim) in a reaction volume of 20 µl. A control reaction lacking the reverse transcriptase enzyme also was prepared for each RNA sample and used in PCR analysis to monitor potential DNA contamination. The first round PCR reaction mixture  
15 consisted of 1 µM each of the outer primers, 1/4 of the RT-reaction volume, and 1U Taq Polymerase (Perkin-Elmer). The PCR reaction was run for 35 cycles: 1.5 minutes at 94°C, 1 minute at 56°C, and 2 minutes at 72°C. For the second PCR round, 1 µl of the first round PCR products were added to the 50 µl reaction mix. The conditions were the same as for the first round except that the annealing  
20 temperature was changed from 56°C to 65°C. RT-PCR was performed 5 to 10 times for each sample from different RNA preparations. Reaction mixtures lacking template were used as negative PCR controls. Samples were normalized by the amplification of β-actin.

#### 5. PCR analysis and Southern blotting

- 25 PCR products were examined by Southern blot analysis. Aliquots (20 µl of the 50 µl PCR reaction) were subjected to gel electrophoresis (1% or 2% agarose gel), stained with ethidium bromide, and blotted onto nylon membranes. PCR products were visualized with a digoxigenin nonradioactive nucleic acid

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- labeling and detection system according to the manufacturer's directions (DIG DNA Labeling Kit and DIG Luminescent Detection Kit for Nucleic Acid, Boehringer Mannheim). Briefly, membranes were prehybridized for 2 hours at 42°C in DIG-easy Hyb buffer (Boehringer Mannheim). The membrane were then
- 5 hybridized overnight at 42°C with a digoxigenin-11-dUTP labeled probe. The probe was a second round PCR product derived from a neuroblastoma cell line. The probe was generated by random primed labeling. Membranes were washed under conditions of low (25°C, 2X SSC, 0.1% SDS, 2 times for 15 minutes each) to high (68°C, 0.1% SSC, 0.1% SDS, 2 times for 15 minutes each) stringency.
- 10 Signal was detected by chemiluminescence (DIG Luminescent Detection Kit for Nucleic Acid, Boehringer Mannheim) by exposing the membrane to high performance luminescence detection film (Hyperfilm™ ECL, Amersham) for 2-3 hours at room temperature.

#### 6. *Sequencing of the second round PCR products*

- 15 PCR products from the second round amplification were purified with the QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions. Purity was assessed by agarose gel electrophoresis and spectrophotometric analysis. Nucleotide sequences of PCR fragments were determined using a dye terminator method (Dye Terminator Cycle Sequencing Kits;
- 20 Perkin-Elmer) in an Applied Biosystem 370 A DNA Sequencer. Sequence reactions were performed on both strands of template DNA. Results were compared to the *mu1* receptor sequence (Genbank n. L25119).

#### 7. *Northern RNA blotting analysis*

- Briefly, 2 µg of poly A<sup>+</sup> RNA for each sample was fractionated in 1% agarose/formaldehyde gel, transferred to nylon membrane, and prehybridized for 2
- 25 hours at 50°C in DIG-easy Hyb buffer (Boehringer Mannheim). The filter was hybridized overnight at 50°C with the same digoxigenin-11-dUTP labeled probe used for Southern blot hybridization. Washes were performed under conditions of

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low (25°C, 2X SSC, 0.1% SDS, 2 times for 15 minutes each) to high (68°C, 0.1% SSC, 0.1% SDS, 2 times for 15 minutes.) stringency. Detection was performed according to manufacturer's instructions (DIG Luminescent Detection Kit for Nucleic Acid, Boehringer Mannheim) and exposure was overnight at room  
5 temperature.

#### 8. Opiate binding analysis

Binding was studied in human granulocytes, normal lung, and non-small cell lung carcinoma tissue. The separate materials were washed and homogenized in 50 volumes of 0.32 M sucrose (pH 7.4) at 4°C. Membrane suspensions were  
10 prepared and the displacement analysis was performed as described in elsewhere (Stefano GB *et al.*, *Proc. Natl. Acad. Sci. USA* 90:11009 (1993); Stefano GB *et al.*, *Proc. Natl. Acad. Sci. USA* 89:9316 (1992); and Stefano GB *et al.*, *J. Biol. Chem.* 270:30290 (1995)). Briefly, aliquots of membrane suspensions from human granulocytes and lung non-small cell carcinoma (less 9% lymphocyte infiltrate)  
15 were incubated with non-radioactive compounds at six concentrations for 10 minutes at 22°C and then with 40 nM of <sup>3</sup>H-dihydromorphine ([<sup>3</sup>H]-DHM) for 60 minutes at 4°C. One hundred percent binding is defined as bound [<sup>3</sup>H]-DHM in the presence of 10 µM dextrophan minus bound [<sup>3</sup>H]-DHM in the presence of 10 µM levorphanol. The IC<sub>50</sub> values are defined as the concentration of drug that  
20 elicits half-maximal inhibition of specific binding. The mean SEM for three experiments is given. The displacement analysis data indicate the potency of various opioid extracts in displacing [<sup>3</sup>H]-DHM and may give specific information on different receptor populations. Incubation medium for Met-enkephalin contained phosphoramidon (100 µM) and bestatin (100 µM) to inhibit enzyme  
25 action.

#### 9. Opioid peptides, opiates, and opioid receptor antagonists

Mu-selective agonists ([D-Ala<sup>2</sup>,N-Me-Phe-Gly<sup>5</sup>(ol)]-enkephalin (DAGO), dihydromorphine, and morphine), delta-selective agonists (enkephalinamide

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(DAMA), D-Ala-d-Leu-enkephalin (DADLE), Tyr-D-Pen-Gly-Phe-D-Pen (DPDPE), deltorphin, and Met-enkephalin), kappa-selective agonist (dynorphin 1-17), and opioid receptor antagonists (naltrexone and naloxone) were purchased from Sigma.

5 10. *Pathological examination*

Lung (tumor and adjacent normal lung) and thyroid tissue was obtained from resections and either frozen immediately in liquid nitrogen, embedded in optimal cutting temperature, and frozen at -70°C, or formalin fixed and paraffin embedded. Frozen tissue was fixed before staining with 1% paraformaldehyde and  
10 stained with hematoxylin and eosin. Lung tissue pathological diagnosis confirmed the presence of poorly differentiated, non-small cell carcinoma with squamoid and clear cell features. Lung not involved with tumor showed edema, other reactive changes, and mild, irregular fibrosis. Lymphocyte infiltration of the tumor was less than 5%. Thyroid tissue showed follicular hyperplasia and random irregular  
15 scarring.

11. *Direct measurement of NO release*

A NO-specific amperometric probe (World Precision Instruments, Sarasota, FL) was used to measure directly the NO released from granulocytes ( $10^7$ /ml), normal lung tissue, and non-small cell lung carcinoma tissue (1 mg wet  
20 weight) as described elsewhere (Stefano GB *et al.*, *J. Biol. Chem.* 270:30290 (1995) and Magazine HL *et al.*, *J. Immunol.* 156:4845 (1996)). The system was calibrated daily using different concentrations of the nitrosothiol donor S-nitroso-N-acetyl-DL-penicillamine (SNAP; Sigma, St. Louis, MO) to generate a standard curve. The concentration of NO gas in solution was measured in real-time with the  
25 DUO 18 computer data acquisition system (World Precision Instruments). Each experiment was simultaneously performed with a control (vehicle alone) to exclude experimental drift in NO release unrelated to the administration of compounds. The morphine-stimulated release of NO from granulocytes was demonstrated to be



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inhibited by naloxone and the NOS inhibitors, N-nitro-L-arginine and N omega-nitro-L-arginine methyl ester (L-NAME).

For cultured endothelial cells ( $10^6$  cells/chamber), NO release also was measured directly using an NO-specific amperometric probe. Briefly, the cells  
5 were placed in a superfusion chamber in 2 mL PBS. A micromanipulator (Zeiss-Eppendorff) attached to the stage of a inverted microscope (Nikon Diaphot) was employed to position the amperometric probe 15  $\mu$ m above the cell surface. The system was calibrated daily using different concentration of the nitrosothiol donor S-nitroso-N-acetyl-DL-penicillamine (SNAP; Sigma, St. Louis, MO) to generate a  
10 standard curve. Baseline levels of NO release were determined by evaluation of NO release in PBS. Cells were stimulated with the respective ligand, and the concentration of NO gas in solution was measured in real-time with the DUO 18 computer data acquisition system (World Precision Instruments). The amperometric probe was allowed to equilibrate for 12 hours in PBS prior to being  
15 transferred to the superfusion chamber containing the cells, and manipulation of the cells was performed only with glass instruments. Each experiment was repeated four times. In addition, each experiment was simultaneously performed with a control (vehicle alone) to exclude experimental drift in NO release unrelated to the administration of compounds.

20 12. *Monitoring intracellular calcium levels*

Human arterial endothelial cells were purchased from Cell Systems (Eugene, Oregon) and grown at 37°C, 5% CO<sub>2</sub> in chamber slides (Nunc Int.) with CS-C medium (Cell Systems) supplemented with 10% fetal calf serum and endothelial growth factor. Intracellular calcium levels were measured by dual  
25 emission microfluorimetry using the fluorescent dye fura-2/AM. Cells were loaded with the fluorescent ion indicator as follows. First, the cells were washed twice in incubation medium without calcium, balanced with sucrose to maintain osmolarity (Stefano GB *et al.*, *Science* 213:928-930 (1981)), and then incubated with 5  $\mu$ M fura-2/AM for 30 minutes at room temperature. The non-ionic and non-denaturing

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detergent Pluronic F-127 helped disperse acetoxymethyl (AM) esters of fura-2 in the loading buffer. Cells were washed twice with PBS and then test drugs were added. The intracellular calcium concentration ( $[Ca^{2+}]_i$ ) was calculated from the fluorescence ratio (340 and 380 nm excitation and 510 nm emission wavelength) according to the following equation:  $[Ca^{2+}]_i = (R - R_{min}) k_d \beta / (R_{max} - R)$ ; where  $R$  = fluorescence ratio recorded from the cell;  $R_{min}$  = fluorescence ratio of fura-2 free acid recorded in absence of  $Ca^{2+}$ ;  $R_{max}$  = fluorescence ratio of fura-2 free acid recorded in saturating concentration of  $Ca^{2+}$ ;  $k_d$  = calcium dissociation constant of the dye; and  $\beta$  = the ratio of fluorescence of fura-2 free acid in the  $Ca^{2+}$  free form to the  $Ca^{2+}$  saturated form recorded at the wavelength used in the denominator of the ratio. Images were acquired every 0.4 seconds with an image processing system COMPIX C-640 SIMCA (Compix Inc., Mars, PA) and an inverted Nikon, Inc., microscope. Experiments were carried out at room temperature in PBS without calcium/magnesium. Receptor antagonists, when used, were administered 2 minutes prior to the respective agonist.

A two-way ANOVA was used for statistical analysis on the peak  $[Ca^{2+}]_i$  time, 7 seconds after agonist exposure to the cells. Each experiment was simultaneously performed with up to 6 cells. The mean value was combined with the mean value taken from 4 other replicates. The SEM represents the variation of the mean of the means.

### 13. Statistical analysis

Data were evaluated using the Student's t-test. Data acquisition for the direct measurement of NO release was by the computer-interfaced DUO-18 software (World Precision Instruments). The experimental values were then transferred to Sigma-Plot and -Stat (Jandel, San Rafael, CA) for graphic representation and evaluation. Data gatherers and were unaware of the experimental treatments.

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Example 2 - mu3 opiate receptor expression

Nested RT-PCR revealed the presence of a mu-specific mRNA having a sequence different from the mu1 receptor sequence in several tissues. First round PCR amplification using the outer primers (SEQ ID NOs:1 and 2) produced a product of about 1450 base pairs from total RNA isolated from a neuroblastoma cell line (SH-SY5Y cell line). This sequence corresponds to the mu1 receptor sequence (Bare LA et al., *FEBS Lett.* 354:213 (1994)). First round PCR amplification using the outer primers, however, produced a larger product from total RNA isolated from human granulocytes or human lung carcinoma. This larger product was about 2000 base pair (2 kb) and was not detected in human thyroid tissue. Controls included samples without reverse transcriptase to monitor genomic contamination and samples without template to monitor PCR contamination.

Second round PCR amplification confirmed that the first round products were related to a mu-specific sequence. Specifically, second round PCR amplification using the inner primers (SEQ ID NOs:3 and 4) produced a product of about 440 base pairs from the samples having the 1450 base pair product (mu1 receptor) and the 2 kb product (mu3 opiate receptor). It is noted that these inner primers correspond to sequences found on a single exon of the mu1 receptor sequence. Since the 1450 base pair product and the larger 2 kb product each contain a similar 440 base pair fragment, the mRNA transcripts from which these products were derived must contain this exon. Again, controls included samples without reverse transcriptase to monitor genomic contamination and samples without template to monitor PCR contamination.

Southern blot analysis using the second round PCR product from the neuroblastoma cell line as probe confirmed that the 1450 base pair product and the larger 2 kb products contain a similar sequence. Specifically, probe hybridized with the first round PCR products from SH-SY5Y cells (1450 base pair product) as well as lung carcinoma and granulocyte cells (2 kb product). In addition, probe hybridized with 2 kb PCR products produced from colon, rectum, skin, lymphnode,

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and breast tissue using the outer primer pair. Similar results were obtained using the following cell lines as sources of total RNA: M14, 2-21, 13443 (melanoma), LoVo, LoVo-R-DOX-1 (colon), OVCA 433 (ovary), CCRF-CEM, CEM-VLB 100, K562 (leukemia), MCF-7, MCF-7ADR/R (breast), DU 145, PC3 (prostate), and T24. The 2 kb product, however, was not consistently detected in lymphnode and breast tissue samples. In addition, the larger 2 kb product was not detected in thyroid or cervix tissue. Likewise, it was not detected in the following cell lines: 2-60, CoLo 205, A-2780, A-2780 Cp8, 5637, and RT4. No probe hybridization was detected in lanes containing the PCR controls (samples without reverse transcriptase and samples without template).

Multiple transcripts were detected by Northern blot analysis using the second round PCR product from the neuroblastoma cell line as probe. The 13.5 kb transcript detected by Raynor K *et al.* (*J. Pharmacol. Exp. Ther.* 272:423 (1995)) in brain tissue was not detected using this probe. Smaller sized transcripts (e.g., 4.5 kb, 2.8 kb, and 1.9 kb), however, were detected. These specific smaller bands were detected in spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood lymphocytes, heart, brain, placenta, liver, skeletal muscle, kidney, and pancreas, but not thyroid.

To determine if the tissues containing the larger 2 kb product express a functional receptor having the pharmacologic properties of a mu3 opiate receptor, the displacement of [<sup>3</sup>H]-DHM binding with mu, delta, and kappa ligands was examined. None of the tested opioid peptides or analogues exhibited binding affinity for the receptors expressed by granulocytes, lung non-small cell carcinoma cells, or normal lung cells (Table 1). The tested opiate alkaloids, however, did exhibit high binding affinity for the receptors expressed by these cells. In addition, thyroid tissue did not exhibit a mu3 displacement profile. Thus, the presence of the larger 2 kb PCR product correlates with the expression of a functional mu3 opiate receptor. In addition, it is likely that the different pharmacological profiles of mu1 and mu3 opiate receptors are due to alternative splicing.

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Table 1. Displacement of [<sup>3</sup>H]-DHM by opioid and non-opioid ligands.

LIGAND	IC <sub>50</sub> nM		
	GRANULOCYTES	LUNG CARCINOMA	NORMAL LUNG
<b>Agonists:</b>			
<i>δ-agonists</i>			
DAMA	>1000	>1000	>1000
Deltorphin	>1000	>1000	>1000
Met-enkephalin	>1000	>1000	>1000
DADLE	>1000	>1000	>1000
DPDPE	>1000	>1000	>1000
<i>μ-agonist</i>			
DAMGO	>1000	>1000	>1000
Dihydromorphine	33 ± 3.9	27 ± 4.2	28.5 ± 3.3
Morphine	29 ± 4.5	29 ± 3.7	28.3 ± 3.1
<i>κ-agonist</i>			
Dynorphin 1-17	>1000	>1000	>1000
<b>Antagonists:</b>			
Naltrexone	30 ± 5.1	31 ± 3.8	33.4 ± 4.1
Naloxone	42 ± 4.7	40 ± 4.1	39.7 ± 3.8

DAMA = (D-Ala<sup>2</sup>,Met<sup>5</sup>)-enkephalinamideDADLE = (D-Ala<sup>2</sup>, Leu<sup>5</sup>)-enkephalinDPDPE = (D-Pen<sup>2</sup>,D-Pen<sup>5</sup>)-enkephalinDAMGO = (Tyr-D-Ala<sup>2</sup>, Gly-N-Me-Phe<sup>4</sup>, Gly(ol)<sup>5</sup>)-enkephalinExample 3 - μ<sub>3</sub> opiate receptor activities

Human granulocytes released NO in response to morphine (1 μM). The NO release was rapid, occurring within 1 minute of exposure. In addition, the NO release was sustained for 10-15 minutes (Figure 1). Pretreatment with naloxone

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blocked the morphine-induced NO release. The mu opioid agonist DAMGO had no effect. The effect of morphine on lung carcinoma tissue was similar to that of granulocytes, however, the NO release was instantaneous (i.e., no latency) and sustained for a greater period of time (greater than 20 minutes). Further, the peak level of NO release was significantly greater (35 nM versus 60 nM;  $P < .005$ ) for lung carcinoma tissue compared to the human granulocytes. Moreover, the release of NO at peak levels (4-12 minutes post exposure) for human granulocytes and non-small cell lung carcinoma tissue were statistically different ( $P < 0.01$ ). Normal lung tissue exhibited about 10 nM NO release which was less than that exhibited by granulocytes upon morphine exposure. Thyroid did not respond to morphine exposure (1  $\mu$ M). Each experiment was repeated 4 times and performed with its own control (vehicle minus drug). The release of NO for these controls was  $0.2 \text{ nM} \pm 1 \text{ SEM}$ .

Pre-treatment with opioid peptides such as DAMA,  $\beta$ -endorphin, or DAMGO (1 nM) prevented morphine-induced NO release from vascular tissue, human monocytes, and human granulocytes. It is noted that DAMA,  $\beta$ -endorphin, and DAMGO at 1 nM significantly ( $P < 0.05$ ) inhibited morphine-induced NO release from vascular tissue at the 4 to 10 minute time points and each time point was replicated four times. For vascular tissue, morphine alone (100 nM) induced the release of  $13.8 \pm 4.1 \text{ nM NO}$  at 4 minutes (Figure 2). DAMA (1 nM) alone failed to induce NO release (0.0 nM NO at 4 minutes). DAMA (1 nM) applied two minutes before morphine exposure (100 nM), however, resulted in the release of only  $5.2 \pm 1.4 \text{ nM NO}$  at 4 minutes post morphine exposure. Naltrindole (1 nM) applied two minutes before DAMA (1 nM), which was applied two minutes before morphine exposure (100 nM), resulted in the release of  $12.0 \pm 3.4 \text{ nM NO}$ . Thus, the inhibition of morphine-induced NO release appeared to be mediated by opioid receptors other than the mu3 opiate receptor since naltrindole (1 nM), a delta opioid receptor antagonist, blocked the inhibition of morphine-induced NO release by DAMA.

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Morphine significantly ( $P < 0.001$ ) induced intracellular calcium level increases in a concentration-dependent manner upon administration to endothelial cells (Table 2). In addition, the morphine-induced increase in  $[Ca^{2+}]_i$  was blocked by prior naloxone exposure (1  $\mu$ M naloxone two minutes prior to morphine exposure). Further, naloxone did not stimulate  $[Ca^{2+}]_i$  at the test concentrations, but did stimulate a non-significant 15% increase at 1 mM. In a representative real-time evaluation of the  $[Ca^{2+}]_i$  stimulated by morphine (1  $\mu$ M), the observed increase started three seconds after morphine exposure to the endothelial cells (Figure 3). In part, the three second time period includes the time required for diffusion of the drug to the receptor since it is applied by bath application. The increase in  $[Ca^{2+}]_i$  lasted for about 100-142 seconds (24 measurements) post drug application (Figure 4). The pseudocolor image assigned by the commercial software revealed bright pink color seven seconds after morphine addition, indicating strong intracellular level increases of calcium. Endothelial cells exposed to naloxone (1  $\mu$ M) prior to morphine exposure resulted in a general absence of the calcium-Fura-2 pink color at seven seconds similar to that observed for endothelial treated with vehicle only.

Table 2. Morphine induced intracellular calcium transients in a concentration dependent and receptor mediated manner.

Treatment	$[Ca^{2+}]_i$ nM
	endothelial cells
Control	$31.2 \pm 6.7$
Morphine (1 $\mu$ M)	$275.4 \pm 21^*$
Morphine (100 nM)	$183 \pm 21^*$
Morphine (10 nM)	$96.4 \pm 16^*$
Morphine (1 nM)	$38.5 \pm 6.6$
Morphine + Naloxone (1 $\mu$ M)	$48.6 \pm 14$

\* represents statistically significant at the  $P < 0.01$  level of confidence.

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DAMGO (1  $\mu$ M), a  $\mu$ 1 and  $\mu$ 2 agonist did not stimulate endothelial  $[Ca^{2+}]_i$  increases (Figure 3). In addition, DADLE, DAMA,  $\beta$ -endorphin, and dynorphin 1-17 in the concentration range of 1 pM to 1  $\mu$ M did not stimulate intracellular calcium level increases in the endothelial cells, indicating  $\mu$ 3 opiate receptor mediation.

In cultured human arterial endothelial cells, morphine induced  $[Ca^{2+}]_i$  increases preceded morphine induced NO release by  $40 \pm 8$  seconds (Figure 4). Morphine addition (1  $\mu$ M) to the medium resulted in immediate  $[Ca^{2+}]_i$  increase (application at base of the steep increase) that was then followed by a progressive decrease lasting about 2 minutes. About 40 seconds later, an increase in NO release (peak level 36 nM for morphine) occurred which lasted for 10-18 minutes. The raw data were graphed and connected with spline curves so that the precise times could be visualized (Figure 4). In addition, the increases in  $[Ca^{2+}]_i$  was linked to the increases in NO release. Over a six hour period, the  $Ca^{++}$ -free incubation medium was changed seven times to leech out intracellular calcium stores. After the six hour incubation, morphine (1  $\mu$ M) increased  $[Ca^{2+}]_i$  to  $41 \pm 3.6$ . This level is substantially lower than that observed under non-leeching conditions (Table 1). Furthermore, NO release was barely above background in the  $Ca^{++}$ -leached endothelial cell following morphine exposure (NO  $1.9 \pm 0.4$  nM compared to a peak value of  $33.8 \pm 3.9$ ), indicating that intracellular  $Ca^{++}$  levels regulate cNOS activity and the calcium originates from  $\mu$ 3 opiate receptor-coupling to  $[Ca^{2+}]_i$ .

In summary, only cells and tissue having the 2 kb PCR product ( $\mu$ 3 splice variant) exhibited a  $\mu$ 3 displacement profile, morphine-induced  $[Ca^{2+}]_i$  increases, and morphine-induced NO release. For example, thyroid tissue was devoid of the 2 kb PCR product ( $\mu$ 3 splice variant) and lacked opiate binding. In human lung cell carcinoma, the opiate-stimulated NO release was significantly higher and prolonged compared to that observed in granulocytes and normal lung. Further, the morphine-stimulated NO release was inhibited by pre-treatment with opioid peptides having specificity for opioid receptors other than  $\mu$ 3.



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It is important to note that in tumors morphine-induced NO release appeared not to be under any feedback regulation. The presence of mu3 opiate receptors on both normal and tumor cells demonstrates the importance of opiates in receptor mediated activities affecting homeostatic mechanisms. In addition, the level and duration of NO release are significantly greater for cancer cells in comparison to normal tissue, indicating the need for a reappraisal of mechanisms involved in opiate tolerance and bioavailability in cancer patients undergoing chronic pain therapy. Moreover, the results presented herein indicate that tumors can use endogenous opiates and NO processes to avoid the response to antigenic challenge. Furthermore, since morphine and related opiate alkaloids are extensively used in chronic cancer therapy, their "new" action must be considered.

As described herein, mu3 opiate receptors are a central target for immunoregulation and inflammatory responses, with morphine being responsible for the suppression of these functions. Thus, morphine plays a specific role in the modulation of cellular responsiveness to immunostimulating molecules.

#### Example 4 - Cannabinoid receptor activities

As described herein, cannabinoid receptors mediate activities similar to the activities mediated by the mu3 opiate receptor. In cultured human arterial endothelial cells loaded with fura 2, both morphine and anandamide induced intracellular calcium transients in a concentration and receptor mediated manner. Further, the calcium transient increase was followed by NO release from these cultured endothelial cells. Thus, rapid signal transduction via intracellular calcium takes place in endothelia in response to both endogenous morphine and cannabinoids.

Anandamide significantly ( $P < 0.001$ ) stimulated endothelial intracellular calcium level increases in a concentration-dependent manner (Table 3). Morphine was found to be more potent than anandamide ( $P < 0.001$  at 1  $\mu\text{M}$  to 10 nM). The  $[\text{Ca}^{2+}]_i$  for anandamide stimulation was about half that found for morphine. The  $[\text{Ca}^{2+}]_i$  increase induced by anandamide was blocked by prior exposure to the

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cannabinoid receptor antagonist SR 171416A (Table 3). Further, SR 171416A did not stimulate  $[Ca^{2+}]_i$ .

Table 3. Anandamide induced intracellular calcium transients in a concentration dependent and receptor mediated manner.

	$[Ca^{2+}]_i$ nM
Treatment	endothelial cells
Control	$31.2 \pm 6.7$
Anandamide (1 $\mu$ M)	$135 \pm 17^*$
Anandamide (100 nM)	$96 \pm 14^*$
Anandamide (1 nM)	$34 \pm 4.2$
Anandamide + SR 141716A	$27.5 \pm 5$

\* represents statistically significant at the  $P < 0.01$  level of confidence.

Anandamide exposure (1  $\mu$ M) resulted in  $[Ca^{2+}]_i$  increases in 3 seconds (Figure 5). This  $[Ca^{2+}]_i$  response lasted about 100-142 seconds post drug application (Figure 4), which was similar to that observed for the morphine induced  $[Ca^{2+}]_i$  increases operating through  $\mu_3$  opiate receptors. The pseudocolor image assigned by the commercial software revealed bright pink color seven seconds after anandamide addition, indicating strong intracellular level increases of calcium. Endothelial cells exposed to SR 171416A (1  $\mu$ M) prior to anandamide exposure resulted in a general absence of the calcium-Fura-2 pink color at seven seconds similar to that observed for endothelial treated with vehicle only.

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In cultured human arterial endothelial cells, anandamide induced  $[Ca^{2+}]_i$  increases preceded anandamide induced NO release (Figure 4). Anandamide addition (1  $\mu$ M) to the medium resulted in immediate  $[Ca^{2+}]_i$  increase (application at base of the steep increase) that was then followed by a progressive decrease lasting about 2 minutes. About 40 seconds later, an increase in NO release (peak level 17 nM for anandamide) occurred which lasted for 10-18 minutes. The raw data were graphed and connected with spline curves so that the precise times could be visualized (Figure 4). In addition, the increases in  $[Ca^{2+}]_i$  was linked to the increases in NO release. Over a six hour period, the  $Ca^{++}$ -free incubation medium was changed seven times to leech out intracellular calcium stores. After the six hour incubation, anandamide (1  $\mu$ M) increased  $[Ca^{2+}]_i$  to  $36.4 \pm 3$ . This level is substantially lower than that observed under non-leeching conditions (Table 3). Further, NO release was barely above background in the  $Ca^{++}$ -leached endothelial cell following anandamide exposure ( $1.6 \pm .06$  nM compared to a peak value of  $17.9 \pm 3.3$ ), indicating that intracellular  $Ca^{++}$  levels regulate cNOS activity and the calcium originates from cannabinoid receptor-coupling to  $[Ca^{2+}]_i$ .

In summary, both morphine and anandamide significantly stimulated cultured endothelial intracellular calcium level increases in a concentration-dependent manner in cells preloaded with fura 2/AM. Morphine is more potent than anandamide (approximately 275 vs. 135 nM  $[Ca^{2+}]_i$ ) and the  $[Ca^{2+}]_i$  for both ligands was blocked by prior exposure of the cells to their respective receptor antagonist (naloxone or SR 171416A). Various opioid peptides did not exhibit this ability, indicating a morphine- $\mu_3$  opiate receptor mediated process for morphine. In comparing the sequence of events concerning morphine's and anandamide's action in stimulating both  $[Ca^{2+}]_i$  and NO production in endothelial cells, intracellular calcium concentration increases were found to precede NO release by about 40 seconds. In addition, the opiate and cannabinoid stimulation of  $[Ca^{2+}]_i$  was attenuated in cells leached of calcium, indicating that intracellular calcium levels regulate cNOS activity. Further, anandamide stimulated a lower peak level of NO release as well as a lower amount of intracellular calcium mobilization than

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morphine, indicating the importance of the calcium-NO coupling. Taken together, the present results demonstrate that within 3 seconds of exposure to mu3 opiate receptor or cannabinoid receptor agonists calcium transients are stimulated, lasting about 120 seconds. Thirty to 60 seconds later, following exposure to these agonists, NO release occurs.

#### Example 5 - Methods and materials for ESR studies

##### *1. Tissue*

Internal thoracic artery (ITA) segments were obtained from patients undergoing elective coronary artery bypass grafting (CABG) for atherosclerotic coronary artery disease. Patients with chronic illnesses, such as diabetes or cancer as well as acute processes (e.g., known infections, trauma), were excluded. In all patients undergoing CABG, the analgesic management included induction with fentanyl (fentanyl citrate adjusted for pH) or sufentanil (N-[-4(methoxymethyl)-1-[2-(2 thienyl) ethyl]-4-piperidinyl]-N-phenylpropanamide 2 hydroxy- 1,2,3 propanetricarboxylate) up to 15 µg/kg. Maintenance was achieved with the same agents. Parts of ITA that were not used in the CABG procedure were stored in an electrolyte solution at 4°C (500 mL plasmalyte with 5000 U heparin and 60 mg papaverine). These specimens were immediately transported on ice to the laboratory for processing. Immediately upon arrival, each blood vessel was washed in phosphate buffered saline (PBS) and cut into 3-mm rings. The rings were then cut longitudinally, placed endothelial side up into a superfusion chamber (Bilfinger TV *et al.*, *Ann. Thorac. Surg.* 63:1063-1069 (1997)), and filled with 2 mL of PBS.

##### *2. ESR agonists and antagonists*

17β-estradiol (E2), E2-conjugated to bovine serum albumin (E<sub>2</sub>-BSA), 17α-estradiol, and tamoxifen were obtained from Sigma (St. Louis, MO) and ICI 182, 780 was obtained from Tocris Cookson Inc. (Ballwin, MO). Various concentrations of 17β-estradiol (100 fM to 100 nM) or 17β-estradiol conjugated to bovine serum albumin (E<sub>2</sub>-BSA) (100 fM to 100 nM of 17β-estradiol) were applied

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to cultured endothelial cells or ITA fragments to determine a concentration curve. For inhibition studies, the cultured endothelial cells and ITA fragments were stimulated with  $17\alpha$ -estradiol alone (1 nM; n = 4); tamoxifen alone (1 nM; n = 4); ICI 182, 780 alone (1 nM; n = 4); tamoxifen (1 nM) plus  $17\beta$ -estradiol (1 nM; n = 4); tamoxifen (1 nM) plus  $E_2$ -BSA (1 nM; n = 4); or ICI 182, 780 (1 nM) plus  $E_2$ -BSA (1 nM; n = 4). In the experiments, tamoxifen and ICI 182, 780 were added to the milieu 5 minutes before  $17\beta$ -estradiol or  $E_2$ -BSA.

### 3 Direct measurement of NO release

NO release from the cultured endothelial cells ( $10^6$  cells/chamber) and ITA fragments (3 mm rings) placed in a superfusion chamber in 2 mL PBS was measured directly using an NO-specific amperometric probe (World Precision Instruments, Sarasota, FL) as described herein. Prior to measuring NO release, the cells were exposed to a concentration gradient of the various ligands. Potential antagonists or the NOS inhibitor, N omega-nitro-L-arginine methyl ester (L-NAME), when used, were administered 5 minutes prior to the various estrogen compounds.

### 4. Monitoring intracellular calcium levels

Intracellular calcium levels in human arterial endothelial cells were monitored as described herein for the mu3 studies.

### Example 6 - ESR activities

NO release was measured in real-time using a NO-specific amperometric probe following stimulation of the ITA fragments either with  $17\beta$ -estradiol or  $E_2$ -BSA. Normally these vessel fragments release low levels of NO (0 to 1 nM NO). In real time,  $17\beta$ -estradiol (1 nM) stimulated the release of NO (16.1 nM peak value) over a 10 minute time period (Figure 6A). This  $17\beta$ -estradiol induced release of NO was inhibited by first exposing the tissue to tamoxifen, an intracellular estrogen receptor blocker. Increasing concentrations of  $17\beta$ -estradiol

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(10 pM to 100 nM) resulted in a dose-dependent increase in NO release with a maximal effect observed at 1 nM (Figure 7). The increase in NO release from both the ITA fragments and the cultured arterial endothelial cells peaked within the 2 minute observation period (Figure 6). 17 $\beta$ -estradiol at 10 pM failed to stimulate a significant increase in NO release. In addition, 17 $\alpha$ -estradiol (1 nM) did not stimulate NO release from either tissue. Tamoxifen (1 nM) significantly diminished ( $P < 0.005$ ) the 17 $\beta$ -estradiol-induced increase in endothelial NO release (Figures 6 and 8).

Moreover, 17 $\beta$ -estradiol stimulated NO release by interacting with a cell surface receptor, not an intracellular nuclear receptor, since E<sub>2</sub>-BSA (1 nM) does not penetrate the cellular membrane due to its size yet stimulated NO release from these tissues within 2 minutes of its application (Figure 8 for cultured endothelial cells; similar results were obtained for the ITA fragments). The E<sub>2</sub>-BSA induced NO release also was blocked by tamoxifen pre-treatment (Figure 8). As with 17 $\beta$ -estradiol, the E<sub>2</sub>-BSA-induced NO release was dose dependent with both the ITA fragments and the cultured arterial endothelial cells (Figure 8). Addition of E<sub>2</sub>-BSA at 10 pM to either tissue failed to stimulate a significant increase in NO release. The median effective concentration (EC<sub>50</sub>) for E<sub>2</sub>-BSA-induced NO release was about 600 pM. Thus, E<sub>2</sub>-BSA is as potent as 17 $\beta$ -estradiol in stimulating NO release from these tissues (Figure 7). In addition, gently scraping the ITA to remove the endothelial lining resulted in tissue that was non-responsive to 17 $\beta$ -estradiol and E<sub>2</sub>-BSA addition. Thus, these compounds were affecting an estrogen endothelial cell membrane receptor.

Further, a NOS inhibitor, L-NAME (100  $\mu$ M), blocked the NO release stimulated by 17 $\beta$ -estradiol or E<sub>2</sub>-BSA in both the ITA fragments and the cultured arterial endothelial cells (Table 4). Moreover, a nuclear estrogen receptor antagonist, ICI 162,780 (1 nM), failed to inhibit 17 $\beta$ -estradiol- or E<sub>2</sub>-BSA-induced NO release, supporting the cell surface location of the estrogen receptor (Figure 9 for cultured endothelial cells, similar results were obtained using the ITA fragments).

Table 4. L-NAME inhibits estrogen stimulated NO release by inhibiting cNOS activity in endothelial cells.

Cells	Estrogen agonist (1 nM)	L-NAME ( $\mu$ M)	NO level (nM $\pm$ SEM)
Cultured endothelial cells	17 $\beta$ -estradiol	0	13.8 $\pm$ 1.2
Cultured endothelial cells	17 $\beta$ -estradiol	100	2.3 $\pm$ 0.8
ITA	17 $\beta$ -estradiol	0	14.6 $\pm$ 2.1
ITA	17 $\beta$ -estradiol	100	2.7 $\pm$ 0.9

In real time, 17 $\beta$ -esterdiol (1 nM) stimulated a rapid increase in intracellular calcium concentration within six seconds (Figure 6B). This increase [Ca<sup>2+</sup>]<sub>i</sub> was blocked by prior tamoxifen (1 nM) exposure, but not by ICI 182, 780 (1  $\mu$ M; Figure 6B). The EC<sub>50</sub> value for 17 $\beta$ -estradiol was 500 pM and the IC<sub>50</sub> value for tamoxifen was 800 pM.

In comparing the sequence of events concerning E<sub>2</sub>-BSA's action in stimulating both [Ca<sup>2+</sup>]<sub>i</sub> and NO production in the cultured endothelial cells, the increase in [Ca<sup>2+</sup>]<sub>i</sub> preceded NO release by 38  $\pm$  9 seconds (from 4 experiments, Figure 10). The addition of E<sub>2</sub>-BSA (1 nM) to the medium resulted in an immediate increase in calcium transients (application at base of the steep increase) followed by a progressive decrease lasting about 2 minutes. About 40 seconds later, an increase in NO release (peak level 18 nM for E<sub>2</sub>-BSA) occurred, lasting for about 10 minutes.

Further, over a 3 hour period, the Ca<sup>++</sup> free incubation medium was changed six times to leach out intracellular calcium stores. After the 3 hour incubation, 17 $\beta$ -estradiol (1 nM) increased [Ca<sup>2+</sup>]<sub>i</sub> to 3.8  $\pm$  0.6. This level was substantially lower than the level observed under non-leaching conditions (Figure

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6). Further, NO release was barely above background in the  $\text{Ca}^{++}$ -leached endothelial cell following  $17\beta$ -estradiol exposure (NO  $1.8 \pm 0.6$  nM compared to a peak value of  $16.0 \pm 2.7$ ), indicating that intracellular  $\text{Ca}^{++}$  levels regulate cNOS activity and that  $\text{Ca}^{++}$  originates from ESR's coupling to  $[\text{Ca}^{2+}]_i$ .

5           In summary,  $17\beta$ -estradiol exposure to ITA endothelia and arterial endothelia in culture stimulated NO release within seconds in a concentration-dependent manner whereas  $17\alpha$ -estradiol had no effect.  $17\beta$ -estradiol conjugated to bovine serum albumin ( $\text{E}_2$ -BSA) also stimulated NO release, indicating mediation by a cell surface receptor. Tamoxifen, an intracellular estrogen receptor  
10           inhibitor, antagonized the action of both  $17\beta$ -estradiol and  $\text{E}_2$ -BSA, whereas ICI 182,780, another selective inhibitor of the intracellular nuclear estrogen receptor had no effect. Thus, tamoxifen is an antagonist of ESR1 and ICI 182,780 is not. In addition,  $17\beta$ -estradiol stimulated release of endothelial NO in a manner  
15           dependent on the initial stimulation of intracellular calcium transients. The effect of  $17\beta$ -estradiol on endothelial NO release was abolished by leaching out the intracellular calcium stores, or by the addition of a cNOS inhibitor, L-NAME. These results indicate that a physiological dose of estrogen can acutely stimulate NO release from human endothelial cells via the activation of an ESR that is coupled to increases in intracellular calcium. This cNOS-derived NO, in turn, can  
20           induce a hyperpolarization of the artery's smooth muscle cells leading to vasodilatation via myogenic tone reduction.

#### OTHER EMBODIMENTS

25           It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.



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**WHAT IS CLAIMED IS:**

1. A method for identifying a mu3 opiate receptor agonist, said method comprising:
  - a) contacting a cell with a test molecule, wherein said cell expresses a mu3 opiate receptor, and wherein said test molecule is a molecule other than morphine or dihydromorphine, and
  - b) determining if said test molecule induces a mu3 opiate receptor-mediated response in said cell in a mu3 opiate receptor-specific manner.
2. The method of claim 1, wherein said cell is a cancer cell.
3. The method of claim 1, wherein said mu3 opiate receptor is a human mu3 opiate receptor.
4. The method of claim 1, wherein said determining step comprises monitoring nitric oxide synthase activity in said cell.
5. The method of claim 4, wherein said monitoring nitric oxide synthase activity comprises detecting nitric oxide release from said cell.
6. The method of claim 5, wherein a nitric oxide-specific amperometric probe is used to detect said nitric oxide release.
7. The method of claim 1, wherein said determining step comprises monitoring intracellular calcium levels within said cell.
8. The method of claim 7, wherein a fluorescent ion indicator is used to monitor said intracellular calcium levels.

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9. The method of claim 8, wherein said fluorescent ion indicator comprises Fura-2.

10. The method of claim 1, wherein said determining step comprises monitoring both nitric oxide synthase activity and intracellular calcium levels in said cell.

11. A method for identifying a mu3 opiate receptor antagonist, said method comprising:

a) contacting a cell with a test molecule and a mu3 opiate receptor agonist, wherein said cell expresses a mu3 opiate receptor, and wherein said test molecule is a molecule other than naloxone or naltrexone, and

b) determining if said test molecule influences induction of a mu3 opiate receptor-mediated response in said cell by said mu3 opiate receptor agonist.

12. The method of claim 11, wherein said cell is a cancer cell.

13. The method of claim 12, wherein said mu3 opiate receptor agonist comprises morphine or dihydromorphine.

14. The method of claim 11, wherein said determining step comprises monitoring nitric oxide synthase activity in said cell.

15. The method of claim 11, wherein said determining step comprises monitoring intracellular calcium levels within said cell.

16. The method of claim 11, wherein said determining step comprises monitoring both nitric oxide synthase activity and intracellular calcium levels in said cell.

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17. A method for identifying a cannabinoid receptor agonist, said method comprising:

a) contacting a cell with a test molecule, wherein said cell expresses a cannabinoid receptor, and wherein said test molecule is a molecule other than anandamide, and

b) determining if said test molecule induces a cannabinoid receptor-mediated response in said cell in a cannabinoid receptor-specific manner.

18. The method of claim 17, wherein said cell is an endothelial cell.

19. The method of claim 17, wherein said cannabinoid receptor is a human cannabinoid receptor.

20. The method of claim 17, wherein said determining step comprises monitoring nitric oxide synthase activity in said cell.

21. The method of claim 20, wherein said monitoring nitric oxide synthase activity comprises detecting nitric oxide release from said cell.

22. The method of claim 21, wherein a nitric oxide-specific amperometric probe is used to detect said nitric oxide release.

23. The method of claim 17, wherein said determining step comprises monitoring intracellular calcium levels within said cell.

24. The method of claim 23, wherein a fluorescent ion indicator is used to monitor said intracellular calcium levels.

25. The method of claim 24, wherein said fluorescent ion indicator comprises Fura-2.

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26. The method of claim 17, wherein said determining step comprises monitoring both nitric oxide synthase activity and intracellular calcium levels in said cell.

27. A method for identifying a cannabinoid receptor antagonist, said method comprising:

a) contacting a cell with a test molecule and a cannabinoid receptor agonist, wherein said cell expresses a cannabinoid receptor, and wherein said test molecule is a molecule other than SR 141716A, and

b) determining if said test molecule influences induction of a cannabinoid receptor-mediated response in said cell by said cannabinoid receptor agonist.

28. The method of claim 27, wherein said cell is an endothelial cell.

29. The method of claim 27, wherein said cannabinoid receptor agonist comprises anandamide.

30. The method of claim 27, wherein said determining step comprises monitoring nitric oxide synthase activity in said cell.

31. The method of claim 27, wherein said determining step comprises monitoring intracellular calcium levels within said cell.

32. The method of claim 27, wherein said determining step comprises monitoring both nitric oxide synthase activity and intracellular calcium levels in said cell.

33. A method for identifying an estrogen surface receptor agonist, said method comprising:

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a) contacting a cell with a test molecule, wherein said cell expresses an estrogen surface receptor, and

b) determining if said test molecule induces an estrogen surface receptor-mediated response in said cell in an estrogen surface receptor-specific manner.

34. The method of claim 33, wherein said cell is an endothelial cell.

35. The method of claim 33, wherein said estrogen surface receptor is a human estrogen surface receptor.

36. The method of claim 35, wherein said estrogen surface receptor is ESR1.

37. The method of claim 33, wherein said test molecule is plasma membrane impermeable.

38. The method of claim 33, wherein said determining step comprises monitoring nitric oxide synthase activity in said cell.

39. The method of claim 33, wherein said determining step comprises monitoring intracellular calcium levels within said cell.

40. The method of claim 33, wherein said determining step comprises monitoring both nitric oxide synthase activity and intracellular calcium levels in said cell.

41. A method for identifying an estrogen surface receptor antagonist, said method comprising:

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a) contacting a cell with a test molecule and an estrogen surface receptor agonist, wherein said cell expresses an estrogen surface receptor, and

b) determining if said test molecule influences induction of an estrogen surface receptor-mediated response in said cell by said estrogen surface receptor agonist.

42. The method of claim 41, wherein said estrogen surface receptor agonist is selected from the group consisting of estrogen,  $17\beta$ -estradiol, and  $17\beta$ -estradiol-BSA.

43. The method of claim 41, wherein said test molecule is plasma membrane impermeable.

44. The method of claim 41, wherein said determining step comprises monitoring nitric oxide synthase activity in said cell.

45. The method of claim 41, wherein said determining step comprises monitoring intracellular calcium levels within said cell.

46. The method of claim 41, wherein said determining step comprises monitoring both nitric oxide synthase activity and intracellular calcium levels in said cell.

47. An isolated nucleic acid molecule comprising first and second nucleic acid sequences, said first nucleic acid sequence being substantially homologous to SEQ ID NO:1, said second nucleic acid sequence being substantially homologous to SEQ ID NO:2, and said first and second nucleic acid sequences being separated by more than about 1500 nucleotides.

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48. The isolated nucleic acid molecule of claim 47, wherein said isolated nucleic acid molecule encodes a mu3 opiate receptor polypeptide.

49. The isolated nucleic acid molecule of claim 47, wherein said isolated nucleic acid molecule comprising a third nucleic acid sequence at least about 80 percent identical to SEQ ID NO:5, said third nucleic acid sequence being located between said first and second nucleic acid sequences.

50. A host cell comprising an isolated nucleic acid molecule, wherein said isolated nucleic acid molecule comprises first and second nucleic acid sequences, said first nucleic acid sequence being substantially homologous to SEQ ID NO:1, said second nucleic acid sequence being substantially homologous to SEQ ID NO:2, and said first and second nucleic acid sequences being separated by more than about 1500 nucleotides.

51. An isolated polypeptide comprising an amino acid sequence at least about 80 percent identical to SEQ ID NO:6, said polypeptide having between 403 and 600 amino acid residues.

52. A method for treating a mammal having cancer, said method comprising administering a mu3 opiate receptor antagonist to said mammal such that a mu3 opiate receptor-mediated response is reduced, wherein said reduction of said mu3 opiate receptor-mediated response promotes anti-tumor activity in said mammal.

53. The method of claim 52, wherein said mammal is a human.

54. The method of claim 52, wherein said cancer is selected from the group consisting of lung cancer, breast cancer, prostate cancer, colon cancer, carcinoma, leukemia, and melanoma.

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55. The method of claim 52, wherein said mu3 opiate receptor-mediated response comprises a change in intracellular calcium concentration in a cell, said cell being in said mammal.

56. The method of claim 55, wherein said cell is a cancer cell.

57. The method of claim 52, wherein said mu3 opiate receptor-mediated response comprises a change in the amount of nitric oxide released from a cell, said cell being in said mammal.

58. The method of claim 52, wherein said method comprises administering a cannabinoid receptor antagonist to said mammal such that a cannabinoid receptor-mediated response is reduced.

59. The method of claim 58, wherein said cannabinoid receptor antagonist is a CB1 receptor antagonist.

60. The method of claim 52, wherein said method comprises administering an estrogen surface receptor antagonist to said mammal such that an estrogen surface receptor-mediated response is reduced.

61. The method of claim 60, wherein said estrogen surface receptor antagonist is an ESR1 antagonist.

62. The method of claim 60, wherein said estrogen surface receptor antagonist is plasma membrane impermeable.

63. The method of claim 62, wherein said estrogen surface receptor antagonist comprises tamoxifen coupled to bovine serum albumin.



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64. A method for treating a mammal having cancer, said method comprising administering a cannabinoid receptor antagonist to said mammal such that a cannabinoid receptor-mediated response is reduced, wherein said reduction of said cannabinoid receptor-mediated response promotes anti-tumor activity in said mammal.

65. A method for treating a mammal having cancer, said method comprising administering an estrogen surface receptor antagonist to said mammal such that an estrogen surface receptor-mediated response is reduced, wherein said estrogen surface receptor antagonist is plasma membrane impermeable, and wherein said reduction of said estrogen surface receptor-mediated response promotes anti-tumor activity in said mammal.

66. A method for treating a mammal having an inflammatory condition, said method comprising administering a mu3 opiate receptor agonist to said mammal such that a mu3 opiate receptor-mediated response is induced, wherein said induction of said mu3 opiate receptor-mediated response promotes anti-inflammatory or immunosuppressive activity in said mammal.

67. The method of claim 66, wherein said inflammatory condition is selected from the group consisting of arthritis, pericarditis, vasculitis, lupus, bronchitis, and phrenitis.

68. The method of claim 66, wherein said method comprises administering a cannabinoid receptor agonist to said mammal such that a cannabinoid receptor-mediated response is induced.

69. The method of claim 66, wherein said method comprises administering an estrogen surface receptor agonist to said mammal such that an estrogen surface receptor-mediated response is induced.

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70. A method for treating a mammal having an inflammatory condition, said method comprising administering a cannabinoid receptor agonist to said mammal such that a cannabinoid receptor-mediated response is induced, wherein said induction of said cannabinoid receptor-mediated response promotes anti-inflammatory or immunosuppressive activity in said mammal.

71. A method for treating a mammal having an inflammatory condition, said method comprising administering an estrogen surface receptor agonist to said mammal such that an estrogen surface receptor-mediated response is induced, wherein said induction of said estrogen surface receptor-mediated response promotes anti-inflammatory or immunosuppressive activity in said mammal.

72. A method for treating a mammal having sepsis, said method comprising administering a mu3 opiate receptor agonist to said mammal such that a mu3 opiate receptor-mediated response is induced, wherein said induction of said mu3 opiate receptor-mediated response reduces septic shock in said mammal.

73. The method of claim 72, wherein said method comprises administering a cannabinoid receptor agonist to said mammal such that a cannabinoid receptor-mediated response is induced.

74. The method of claim 72, wherein said method comprises administering an estrogen surface receptor agonist to said mammal such that an estrogen surface receptor-mediated response is induced.

75. A method for treating a mammal having sepsis, said method comprising administering a cannabinoid receptor agonist to said mammal such that a cannabinoid receptor-mediated response is induced, wherein said induction of said cannabinoid receptor-mediated response reduces septic shock in said mammal.

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76. A method for treating a mammal having sepsis, said method comprising administering an estrogen surface receptor agonist to said mammal such that an estrogen surface receptor-mediated response is induced, wherein said induction of said estrogen surface receptor-mediated response reduces septic shock in said mammal.

77. A method for treating a mammal having a viral infection, said method comprising administering a mu3 opiate receptor agonist to said mammal such that a mu3 opiate receptor-mediated response is induced, wherein said induction of said mu3 opiate receptor-mediated response promotes an anti-viral response in said mammal.

78. The method of claim 77, wherein said viral infection is an HIV infection.

79. The method of claim 77, wherein said method comprises administering a cannabinoid receptor agonist to said mammal such that a cannabinoid receptor-mediated response is induced.

80. The method of claim 77, wherein said method comprises administering an estrogen surface receptor agonist to said mammal such that an estrogen surface receptor-mediated response is induced.

81. A method for treating a mammal having a viral infection, said method comprising administering a cannabinoid receptor agonist to said mammal such that a cannabinoid receptor-mediated response is induced, wherein said induction of said cannabinoid receptor-mediated response promotes an anti-viral response in said mammal.

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82. A method for treating a mammal having a viral infection, said method comprising administering an estrogen surface receptor agonist to said mammal such that an estrogen surface receptor-mediated response is induced, wherein said induction of said estrogen surface receptor-mediated response promotes an anti-viral response in said mammal.

83. A method for treating a mammal having cardiovascular disease, said method comprising administering an estrogen surface receptor agonist to said mammal such that an estrogen surface receptor-mediated response is induced, wherein said estrogen surface receptor agonist is plasma membrane impermeable.

84. The method of claim 83, wherein said induction of said estrogen surface receptor-mediated response reduces or prevents atherosclerosis in said mammal.

85. A method for treating a mammal with a  $\mu_3$  opiate receptor agonist such that  $\mu_3$  opiate receptor-mediated nitric oxide release is suppressed, said method comprising administering an opioid receptor agonist to said mammal, wherein said opioid receptor agonist is not a  $\mu_3$  opiate receptor agonist, followed by administering said  $\mu_3$  opiate receptor agonist.

86. The method of claim 85, wherein said opioid receptor agonist is selected from the group consisting of DAMA,  $\beta$ -endorphin, and DAMGO.

87. The method of claim 85, wherein said  $\mu_3$  opiate receptor agonist comprises morphine or dihydromorphine.

88. A pharmaceutical formulation comprising a  $\mu_3$  opiate receptor antagonist and an estrogen surface receptor antagonist.

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89. A pharmaceutical formulation comprising a mu3 opiate receptor antagonist and a cannabinoid receptor antagonist.
90. A pharmaceutical formulation comprising an estrogen surface receptor antagonist and a cannabinoid receptor antagonist.
91. A pharmaceutical formulation comprising an estrogen surface receptor antagonist, said estrogen surface receptor antagonist being membrane impermeable.
92. A pharmaceutical formulation comprising a mu3 opiate receptor agonist and an estrogen surface receptor agonist.
93. A pharmaceutical formulation comprising a cannabinoid receptor agonist and an estrogen surface receptor agonist.
94. The use of a mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor antagonist in the manufacture of a medicament for the treatment of cancer, wherein said estrogen surface receptor antagonist is plasma membrane impermeable.
95. The use of a mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor agonist in the manufacture of a medicament for the treatment of an inflammatory condition.
96. The use of a mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor agonist in the manufacture of a medicament for the treatment of sepsis.

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97. The use of a mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor agonist in the manufacture of a medicament for the treatment of a viral infection.

98. The use of an estrogen surface receptor agonist in the manufacture of a medicament for the treatment of cardiovascular disease, wherein said estrogen surface receptor agonist is plasma membrane impermeable.

99. An article of manufacture, comprising packaging material and a mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor antagonist contained within said packaging material, wherein said packaging material comprises a label or package insert indicating that said mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor antagonist can be administered to a mammal to treat cancer, and wherein said estrogen surface receptor antagonist is plasma membrane impermeable.

100. An article of manufacture, comprising packaging material and a mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor agonist contained within said packaging material, wherein said packaging material comprises a label or package insert indicating that said mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor agonist can be administered to a mammal to treat an inflammatory condition.

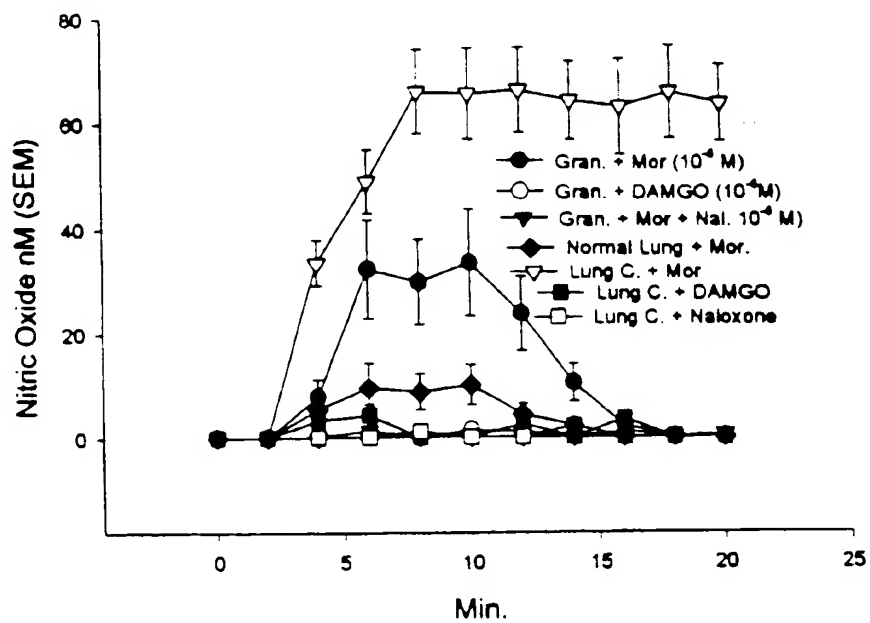
101. An article of manufacture, comprising packaging material and a mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor agonist contained within said packaging material, wherein said packaging material comprises a label or package insert indicating that said mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor agonist can be administered to a mammal to reduce septic shock.

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102. An article of manufacture, comprising packaging material and a mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor agonist contained within said packaging material, wherein said packaging material comprises a label or package insert indicating that said mu3 opiate receptor, cannabinoid receptor, or estrogen surface receptor agonist can be administered to a mammal to treat a viral infection.

103. An article of manufacture, comprising packaging material and an estrogen surface receptor agonist contained within said packaging material, wherein said packaging material comprises a label or package insert indicating that said estrogen surface receptor agonist can be administered to a mammal to treat cardiovascular disease, and wherein said estrogen surface receptor agonist is plasma membrane impermeable.

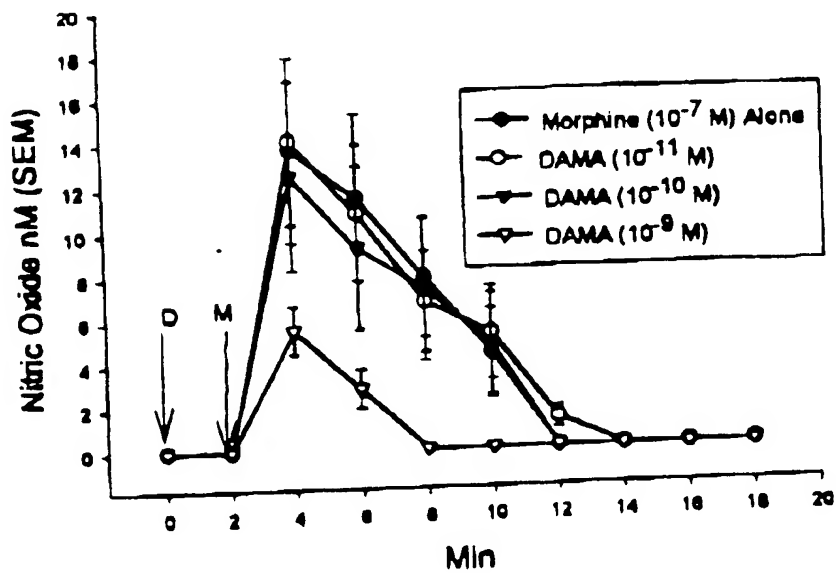
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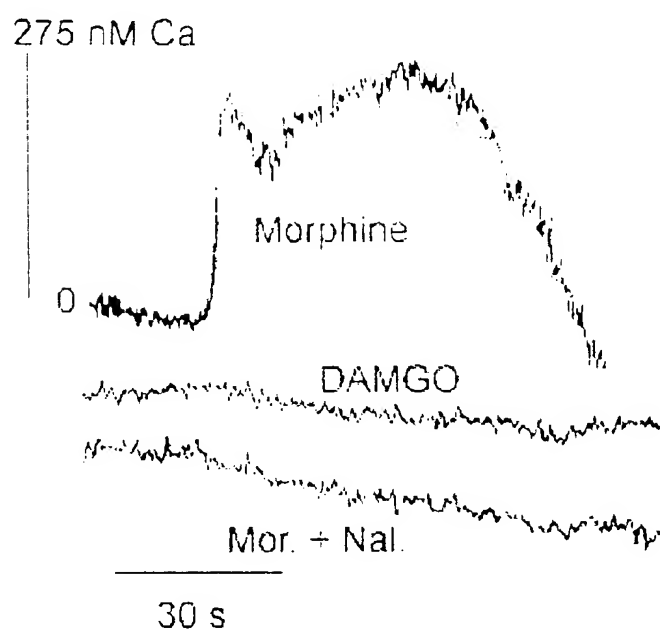


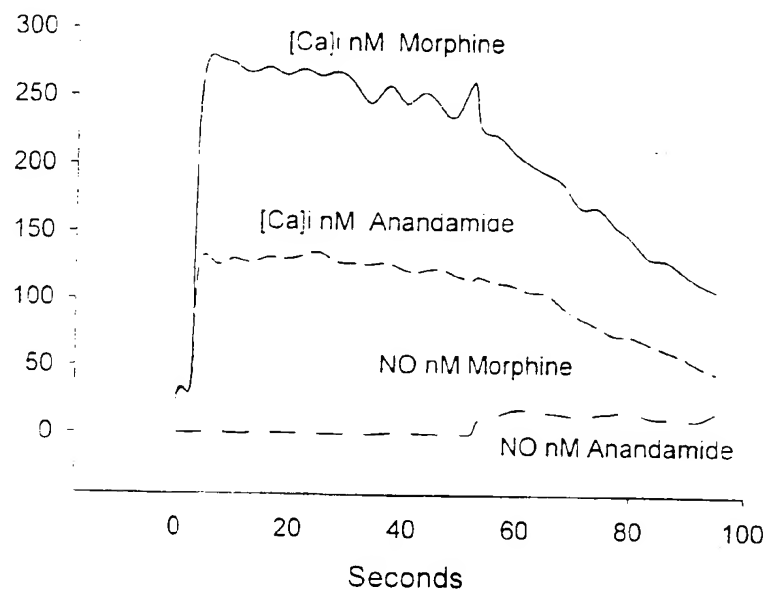


2/10

Figure 2



**Figure 3**

**Figure 4**

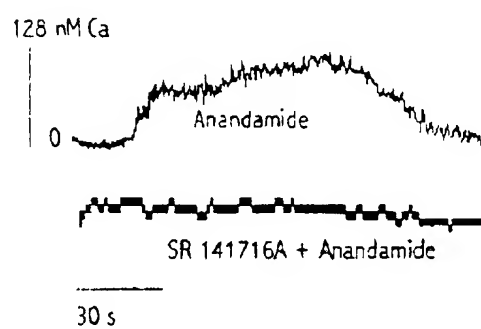
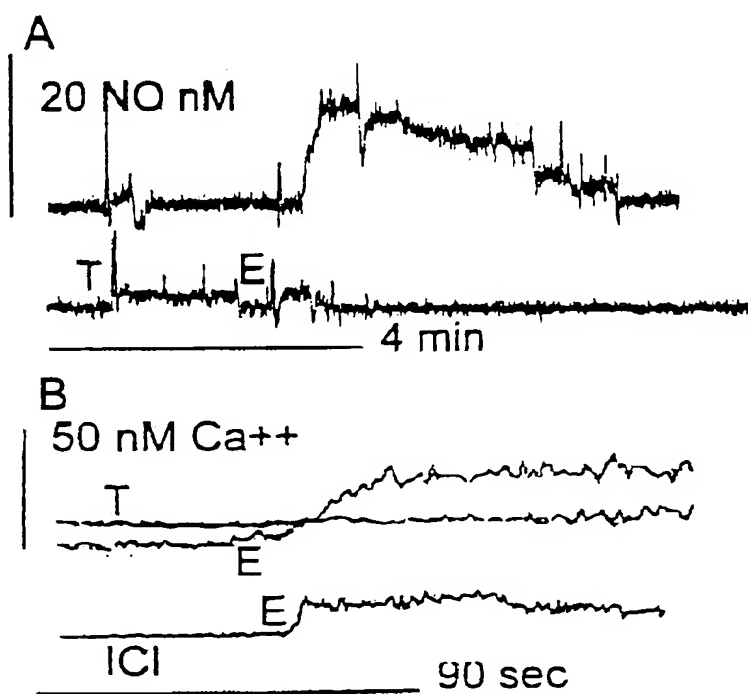
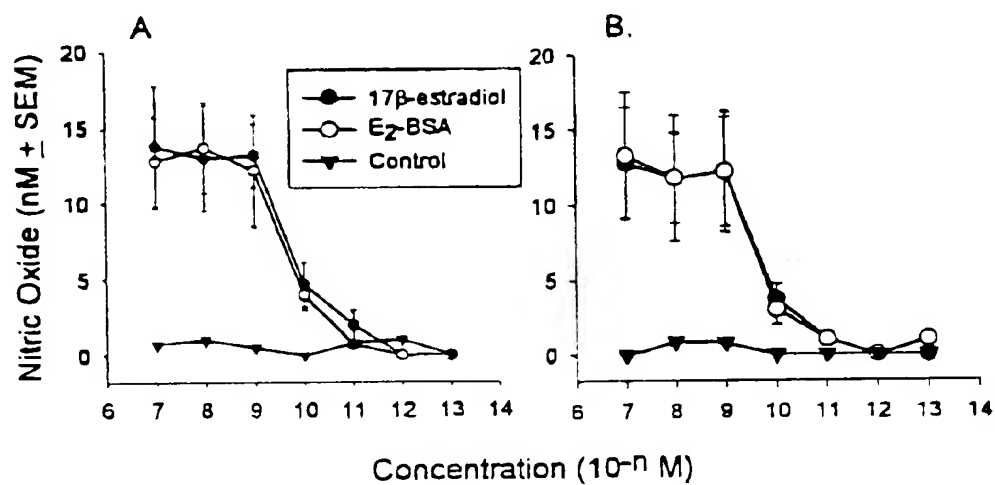
**Figure 5**

Figure 6



**Figure 7** 7/10

8/10

Figure 8

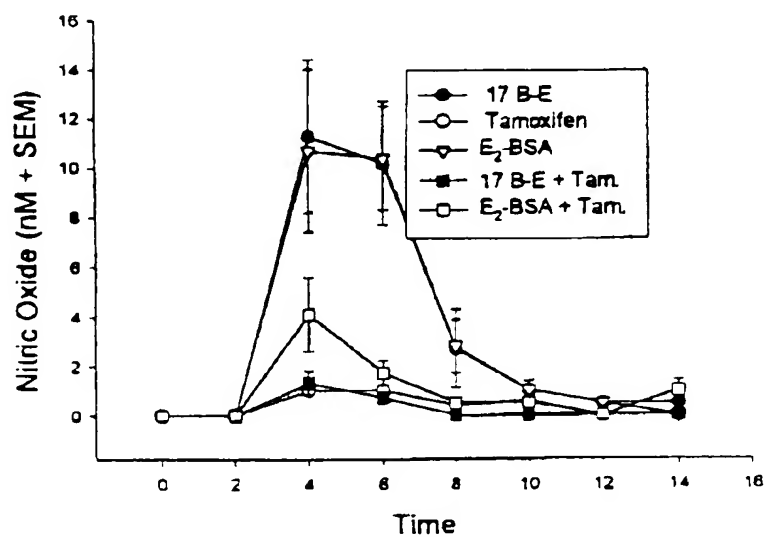


Figure 9

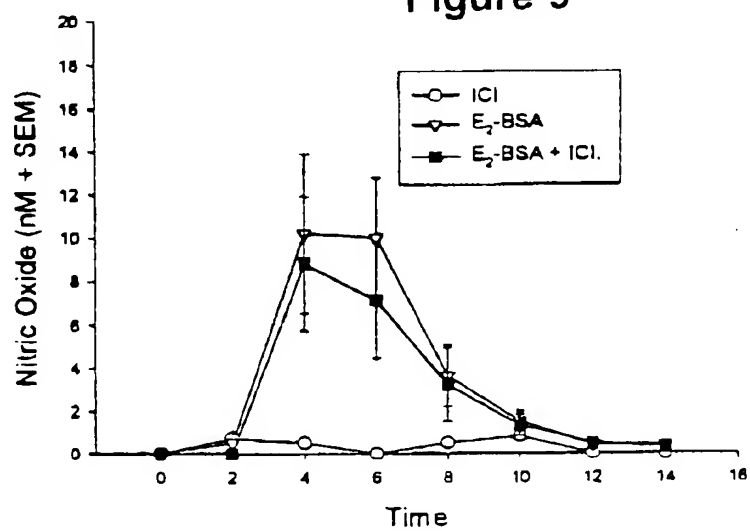
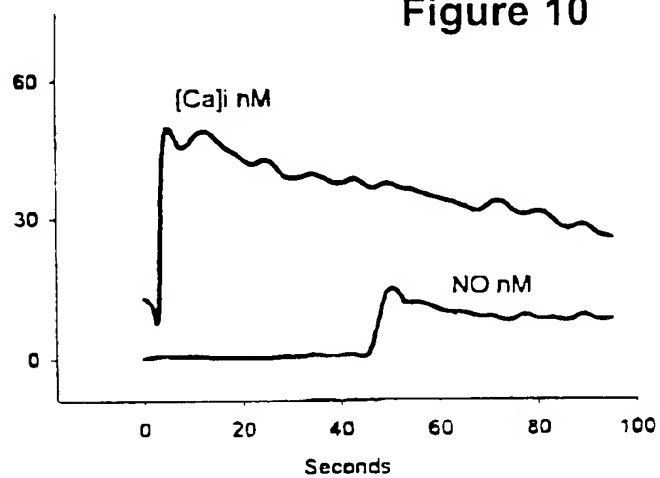




Figure 10



## SEQUENCE LISTING

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/23944

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 14/705; C12N 15/42; C12Q 1/02; A61K 31/00

US CL : 435/7.2, 69.1; 514/2; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.2, 69.1; 514/2; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	IUVONE et al. Opioids Inhibit the Induction of Nitric Oxide Synthase in J774 Macrophages. Biochemical and Biophysical Research Communications. 26 December 1995, Vol. 212, No. 3, pages 975-980, especially pages 976 and 977.	1-10, 66-69, 95
Y	ZHAO et al. Nitric Oxide Synthase Inhibition Attenuates Tolerance to Morphine but not to [D-Ala <sup>2</sup> , Glu <sup>4</sup> ]Deltorphin II, a $\delta_2$ -Opioid Receptor Agonist in Mice. Peptides. 1996, Vol. 17, No. 4, pages 619-623, especially pages 620-622.	1-10, 66-69 95

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents	*7* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

08 FEBRUARY 1999

Date of mailing of the international search report

02 MAR 1999

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
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Washington, D.C. 20231

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Authorized officer

SALLY TENG

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/US98/23944

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FOX-THRELKELD et al. Identification of Mechanisms and Sites of Actions of Mu and Delta Opioid Receptor Activation in the Canine Intestine. The Journal of Pharmacology and Experimental Therapeutics. 1994, Vol. 268, No. 2, pages 689-700, especially pages 691-695.	1-10, 66-69, 95
A	US 5,225,440 A (LONDON et al.) 06 July, 1993, entire document.	1-10, 66-69, 95

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/23944**Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:  
1-10, 66-69, and 95
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/23944

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, Biosis, Medline, WPI

search terms: mu opioid receptor, mu3 opioid receptor, estrogen receptor, cannabinoid receptor, agonist, antagonist, nitric synthase, intercellular calcium.

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-10, drawn to a method of identifying mu3 opiate receptor agonist.  
Group II, claims 11-16, drawn to a method for identifying mu3 opiate receptor antagonist.  
Group III, claims 17-26, drawn to a method of identifying cannabinoid receptor agonist.  
Group IV, claims 27-32, drawn to a method of identifying cannabinoid receptor antagonist.  
Group V, claims 33-40, drawn to a method of identifying estrogen surface receptor agonist.  
Group VI, claims 41-46, drawn to a method of identifying estrogen receptor antagonist.  
Group VII, claims 47-51, drawn to nucleic acid and the encoded polypeptide.  
Group VIII, claims 52-57, 94, drawn to a method of treating cancer using the mu3 opiate receptor antagonist.  
Group IX, claims 58, 59, 64, and 94, drawn to a method of treating cancer using cannabinoid receptor antagonist.  
Group X, claims 60-63, 65, and 94, drawn to a method of treating cancer using estrogen surface receptor antagonist.  
Group XI, claims 66-69, and 95, drawn to a method of treating inflammatory condition using mu3 opiate receptor agonist.  
Group XII, claims 70 and 95, drawn to a method of treating inflammatory condition using cannabinoid receptor agonist.  
Group XIII, claims 71 and 95, drawn to a method of treating inflammatory conditions using estrogen receptor agonist.  
Group XIV, claims 72-74, and 96, drawn to a method of treating sepsis using mu3 opiate receptor agonist.  
Group XV, claims 75 and 96, drawn to a method of treating sepsis using cannabinoid receptor agonist.  
Group XVI, claims 76 and 96, drawn to a method of treating sepsis using estrogen surface receptor agonist.  
Group XVII, claims 77, 78, and 97, drawn to a method of treating viral infection using mu3 opiate receptor agonist.  
Group XVIII, claims 79, 81, and 97, drawn to a method of treating viral infection using cannabinoid receptor agonist.  
Group XIX, claims 80, 82, and 97, drawn to a method of treating viral infection using estrogen surface receptor agonist.  
Group XX, claims 83, 84, and 98, drawn to a method of treating cardiovascular disease using estrogen surface receptor agonist.  
Group XXI, claims 85-87, drawn to a method of treatment associated with suppression of nitric oxide release using mu3 opiate receptor agonist.  
Group XXII, claims 88, 89, and 99, drawn to a pharmaceutical formulation comprising mu3 opiate receptor antagonist.  
Group XXIII, claims 90, 91, and 99, drawn to a pharmaceutical formulation comprising estrogen surface receptor antagonist.  
Group XXIV, claims 92, 93, and 100-103 drawn to a pharmaceutical formulation comprising estrogen surface receptor agonist.

Group XXV, claims 100-102, drawn to a mu3 opiate receptor agonist.

Group XXVI, claims 100-102, drawn to a cannabinoid receptor agonist.

Claims 94-97 and 99-102 are in multiple groups because they encompass multiple inventions. For example, claim 94 requires the use of mu3 opiate receptor antagonist, cannabinoid receptor antagonist, or estrogen surface receptor antagonist in the treatment of cancer.

The inventions listed as Groups I-XVI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical features of Groups I and II are the use of a cell expressing a mu opiate receptor for identifying a mu opiate receptor agonist and a mu opiate receptor antagonist, respectively. The special technical features of Groups III and IV, are the use of a cell expressing a cannabinoid receptor for identifying a cannabinoid receptor agonist and a cannabinoid receptor antagonist, respectively. The special technical features of Groups V and VI are the use of a cell that expresses an estrogen surface receptor for identifying an estrogen surface receptor agonist and an estrogen receptor antagonist,

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respectively. The special technical feature of Group VII is the nucleic acid and amino acid sequences of the mu opiate receptor. The special technical features of Groups VIII, IX, and X are the use of mu opiate receptor antagonist, cannabinoid receptor antagonist, and estrogen surface receptor antagonist, respectively, to treat cancer. The special technical features of Groups XI, XII, and XIII are the use of mu opiate receptor agonist, cannabinoid receptor agonist, or estrogen surface receptor agonist, respectively, to treat inflammatory condition. The special technical features of Groups XIV, XV, and XVI are the use of mu opiate receptor agonist, cannabinoid receptor agonist, and estrogen surface receptor agonist, respectively to treat sepsis. The special technical features of Groups XVII, XVIII, and XIX are the use of mu opiate receptor agonist, cannabinoid receptor agonist, and estrogen surface receptor agonist, respectively, to treat viral infection. The special technical feature of Group XX is the use of estrogen surface receptor agonist to treat cardiovascular disease. The special technical feature of Group XXI is the use of mu opiate receptor agonist for suppressing nitric oxide release. The special technical feature of Group XXII is a pharmaceutical formulation comprising mu opiate receptor antagonist. The special technical feature of Group XXIII is a pharmaceutical formulation comprising an estrogen surface receptor antagonist. The special technical feature of Group XXIV is a pharmaceutical formulation comprising an estrogen surface receptor agonist. The special technical feature of Group XXV is the mu opiate receptor agonist. The special technical feature of Group XXVI is the cannabinoid receptor agonist. The special technical feature of each group is not the same or does not correspond to the special technical feature of any other group because the claimed methods require different starting material and method steps for achieving different goals. The Groups are not linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.